



US005547853A

United States Patent [19][11] **Patent Number:** **5,547,853****Wallner et al.**[45] **Date of Patent:** **Aug. 20, 1996**[54] **CD2-BINDING DOMAIN OF LYMPHOCYTE FUNCTION ASSOCIATED ANTIGEN 3**[75] Inventors: **Barbara P. Wallner**, Cambridge; **Glenn T. Miller**, Haverhill; **Margaret D. Rosa**, Winchester, all of Mass.[73] Assignee: **Biogen, Inc.**, Cambridge, Mass.[21] Appl. No.: **940,861**[22] PCT Filed: **Mar. 12, 1992**[86] PCT No.: **PCT/US92/02050**§ 371 Date: **Oct. 21, 1992**§ 102(e) Date: **Oct. 21, 1992**[87] PCT Pub. No.: **WO92/16622**PCT Pub. Date: **Oct. 1, 1992****Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 770,967, Oct. 7, 1991, abandoned, which is a continuation-in-part of Ser. No. 667,971, Mar. 12, 1991, abandoned.

[51] **Int. Cl.**⁶ **C07K 14/705**; C12N 15/12; C12N 15/62[52] **U.S. Cl.** **435/69.1**; 435/69.7; 435/252.3; 435/320.1; 435/810; 530/350; 436/86; 514/2; 536/23.4; 536/23.5[58] **Field of Search** 435/69.1, 69.7, 435/252.3, 320.1, 810; 530/350, 300, 324-328; 436/501, 86; 536/23.5, 23.4; 206/828[56] **References Cited****U.S. PATENT DOCUMENTS**

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Polypeptides and proteins comprising the CD2-binding domain of LFA-3 are disclosed. DNA sequences that code on expression for those polypeptides and proteins, methods of producing and using those polypeptides and proteins, and therapeutic and diagnostic compositions are also disclosed. Deletion mutants unable to bind CD2 and methods for their use are also disclosed. In addition, fusion proteins which comprise the CD2-binding domain of LFA-3 and a portion of a protein other than LFA-3, DNA sequences encoding those fusion proteins, methods for producing those fusion proteins, and uses of those fusion proteins are disclosed.

35 Claims, 31 Drawing Sheets

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FIG. 1A-1

1 CGACGAGCCATGGTTGCTGGGAGCGACGGGGCCCTGGGGTCCCT 50
MetValAlaGlySerAspAlaGlyArgAlaLeuGlyValLeu
-28

51 CAGCGTGGTCTGCCCTGCTGCTTGGTTTCATCAGCTGTTTTCCC 100
uSerValValCysLeuLeuHisCysPheGlyPheIleSerCysPheSerG
+1

101 AACAAATATATGGTGTGTATGGGAATGTAACCTTCCATGTACCAAGC 150
InGlnIleTyrGlyValValTyrGlyAsnValThrPheHisValProSer
M53

151 AATGTGCCCTTTAAAGAGAGGTCCCTATGGAA~~AA~~CAAAAGGATAAAGTTGC 200
AsnValProLeuLysGluValLeuTrpLysLysGlnLysAspLysValAla
M54 M55

201 AGAACTGGAA~~AA~~ATTCGAATTCAGAGCTTCTCATCTTTTAA~~AA~~ATAGGG 250
aGluLeuGluAsnSerGluPheArgAlaPheSerSerPheLysAsnArgV
M56

251 TTTATTTAGACACTGTGTCAGGTAGCCTC~~ACT~~ATCTACA~~ACT~~TAA~~CA~~TCA 300
aTyrLeuAspThrValSerGlySerLeuThrIleTyrAsnLeuThrSer
M57 M58

301 TCAGATGAAGATGAGTATGAAATGGAATCGCCAAATATTACTGATACCAT 350
SerAspGluAspGluTyIGluMetGluSerProAsnIleThrAspThrMe 86
M59 M60

351 GAAGTCTTCTTATGTGCTTGAGTCTCTCCATCTCCACACTAACTT 400
tLysPhePheLeuTyrValLeuGluSerLeuProSerProThrLeuThrc 103
M61

401 GTGCATTGACTAATGGAAGCATTGAAGTCCAATGCATGATACCAGAGCAT 450
ysAlaLeuThrAsnGlySerIleGluValGlnCysMetIleProGluHis 119
M62 M63

451 TACAACAGCCATCGAGGACTTATAATGTACTCATGGGATTGTCCATGGA 500
TyrAsnSerHisArgGlyLeuIleMetTyrSerTrpAspCysProMetGl 136
M64 M65

501 GCAATGTAACGTAACCTCAACCAGTATATATTTAAGATGGAAATGATC 550
uGlnCysLysArgAsnSerThrSerIleTyrPheLysMetGluAsnAspL 153
M66

FIG. 1A-2

FIG. 1B

551	TTCACAAAATAACAGTGACTCTTAGCAATCCATTATTATAACAACA <u>euProGlnLysIleGlnCysThrLeuSerAsnProLeuPheAsnThrThr</u>	600 169
	M90	
	M91	
601	TCATCAATCATTTTGACAACCTGTATCCCAAGCAGCGGTCAATCAAGACA <u>SerSerIleIleLeuThrThrCysIleProSerSerGlyHisSerArgHi</u>	650 186
	M92	
651	CAGATATGCACTTATACCCATACCATTAGCAGTAATTACAACATGTATTG sArgTyraAlaLeuIleProIleProLeuAlaValIleThrThrCysIleV Transmembrane	700 203
701	TGCTGTATATGAATGGTATTTCTGAAATGTGACAGAAACCAGACAGAACC aIleuTyrrMetAsnGlyIleLeuLysCysaspArgLysProaspArgThr	750 219
751	AACTCCAATTGATTGGTAACAGAGAAGATGAAGACACAGCATAACTAAATT AsnSerAsn	800 222
801	ATTTTAAAACATAAAAAGCCATCTGATTTCTCATTTGAGTATTACAATT T	850
851	TTGAACAACACTGTTGGAAATGTAACTTGAAGCAGCTGCTTTAAGAAGAAAT	900
901	ACCCACTAACAAAGAACAAGCATTAGTTTGGCTGTCATCAACTTATTAT	950
951	ATGACTAGGTGCTTGTCTTTTGTGTCAGTAAATGTTTACTGATGATG	1000
1001	TAGATACTTTTGTAAATAAATGTAATATGTACACAAGTG	1040

FIG. 2A

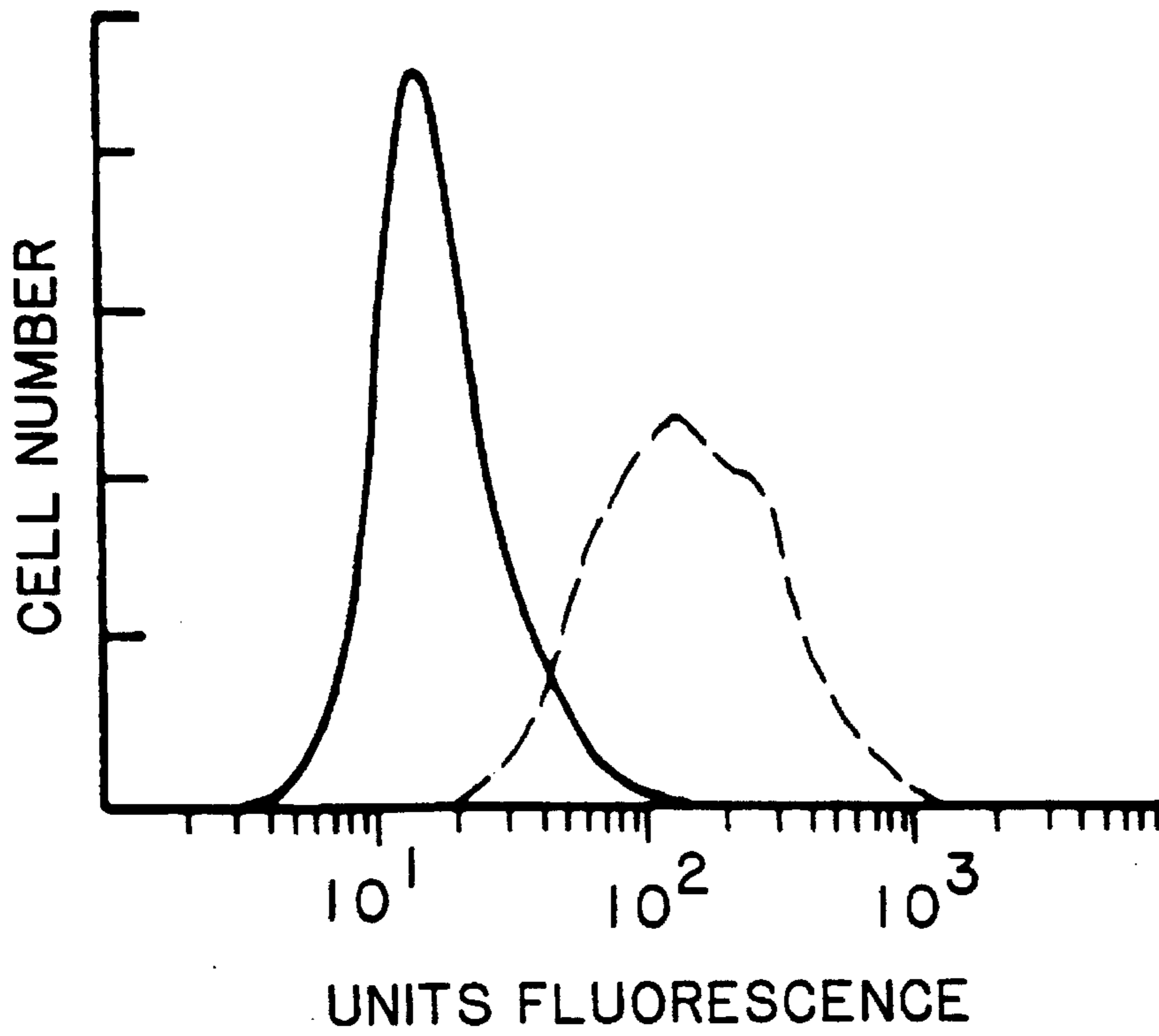


FIG. 2B

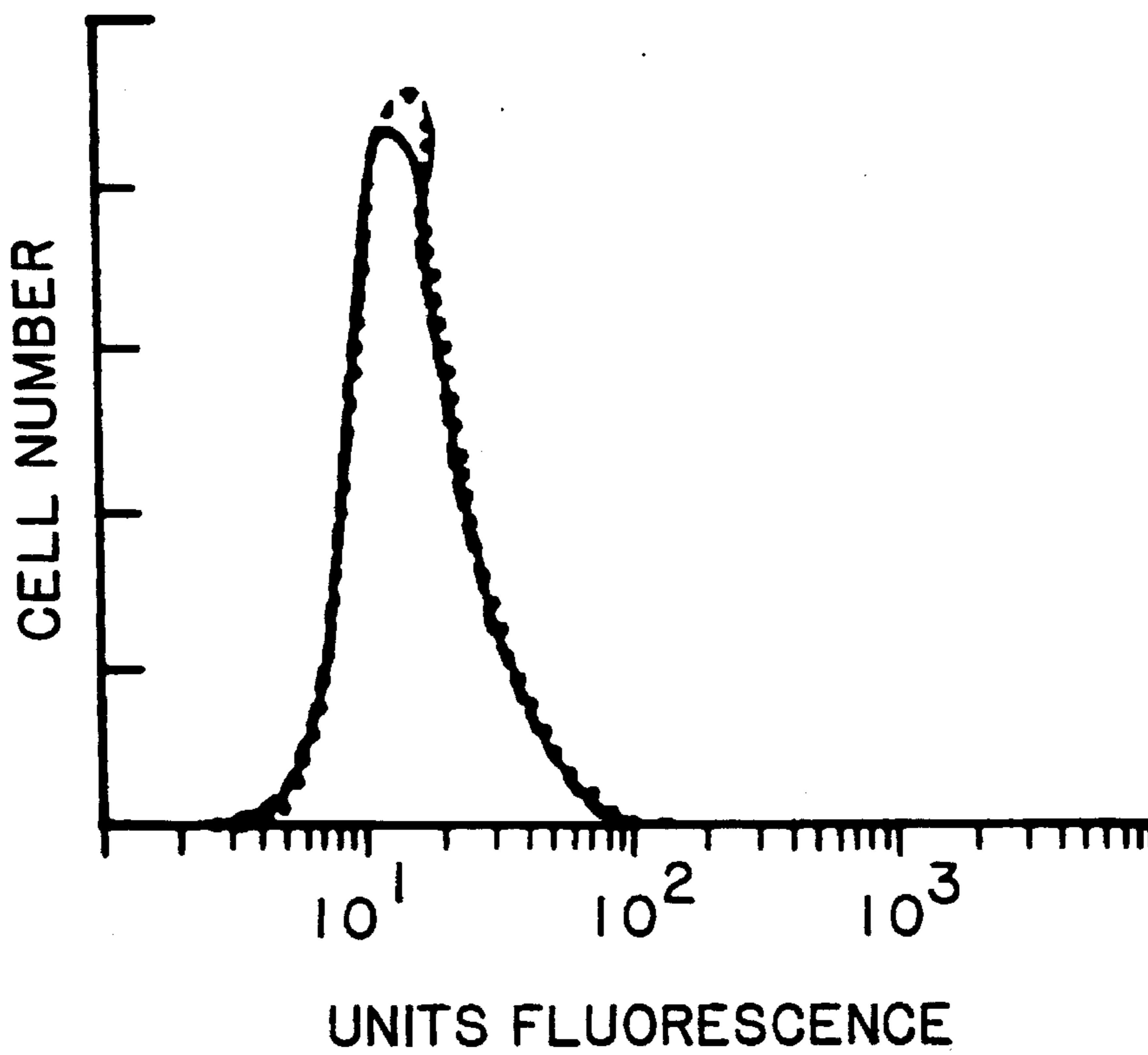


FIG. 2C

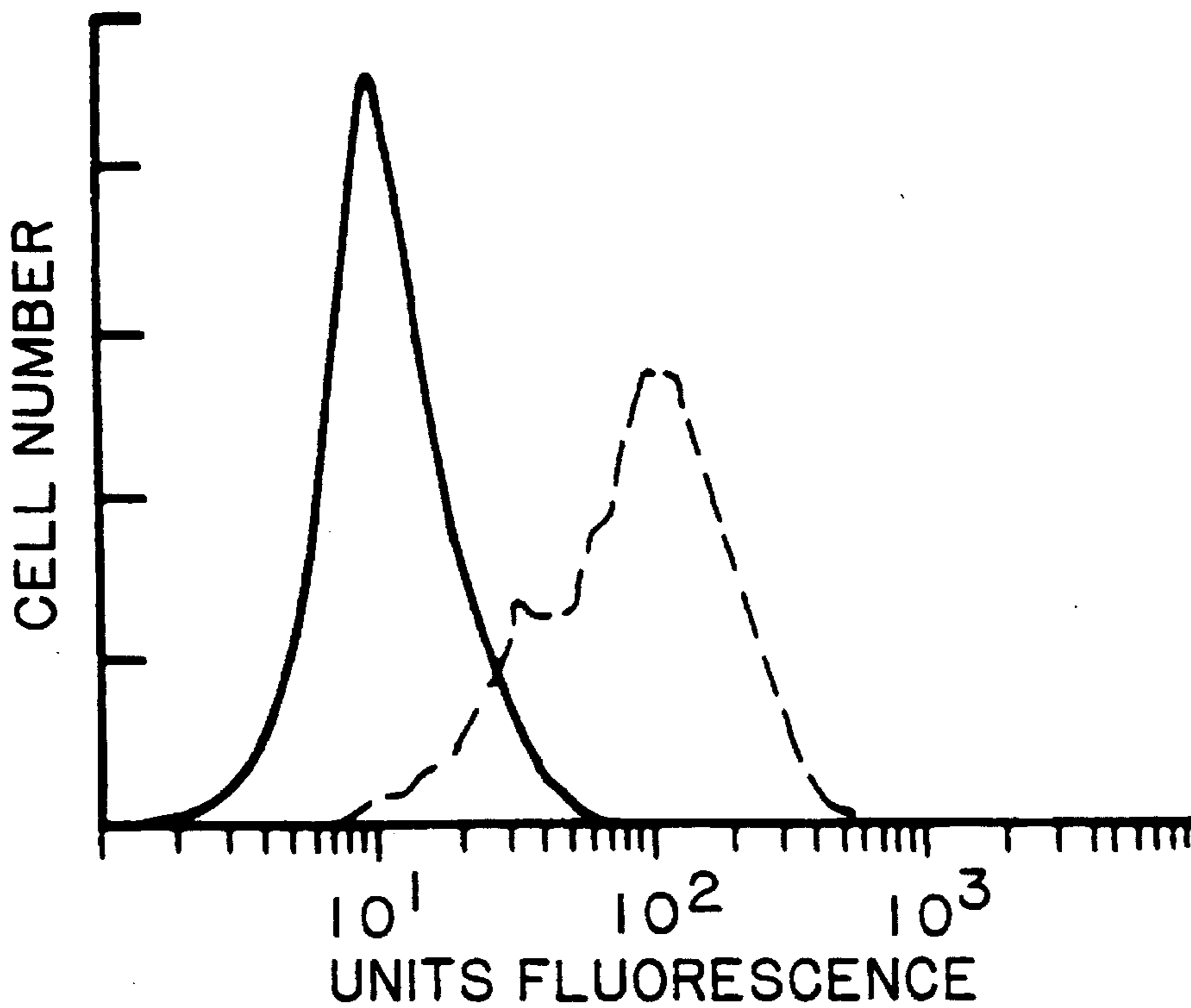


FIG. 2D

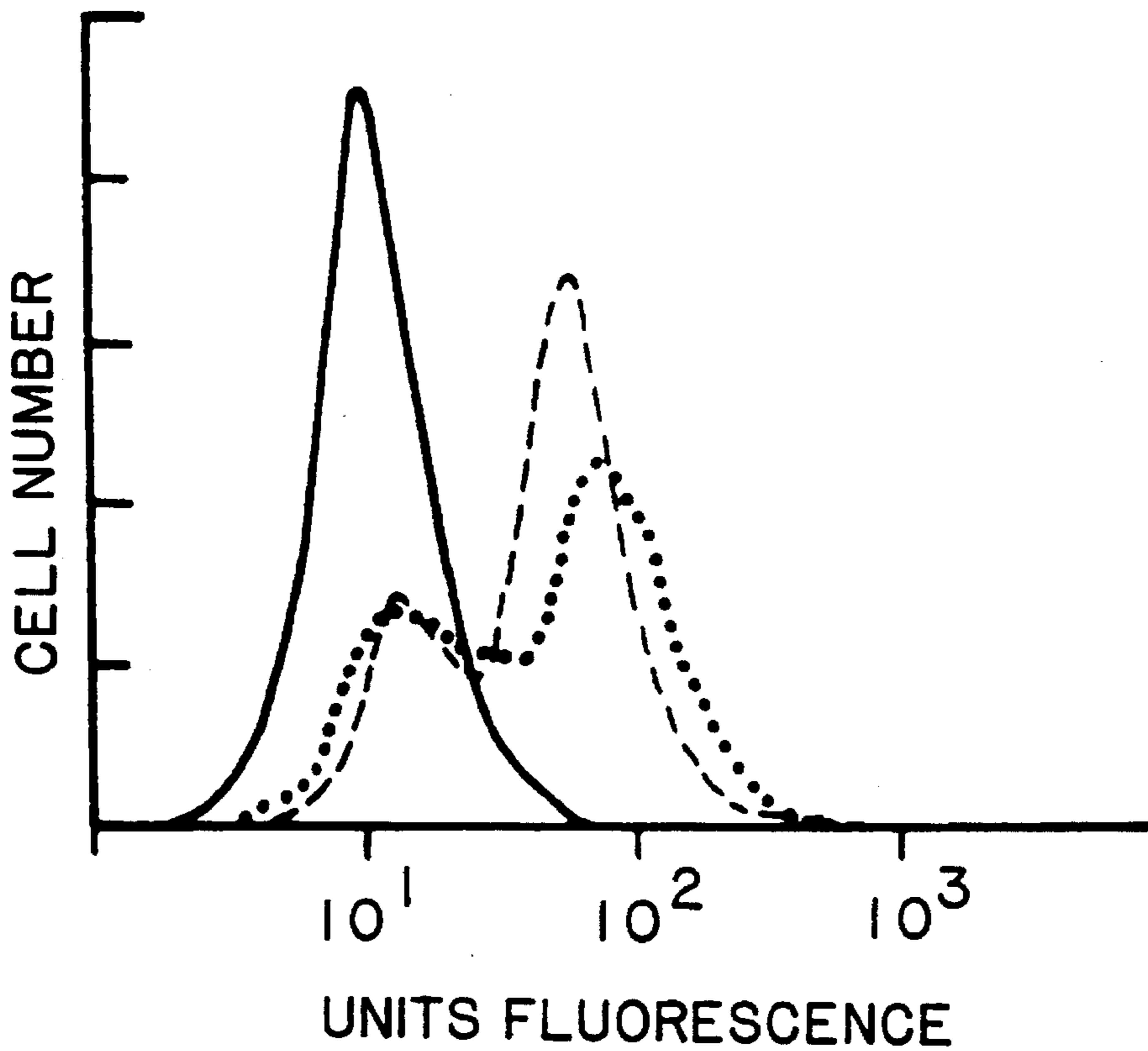


FIG. 2E

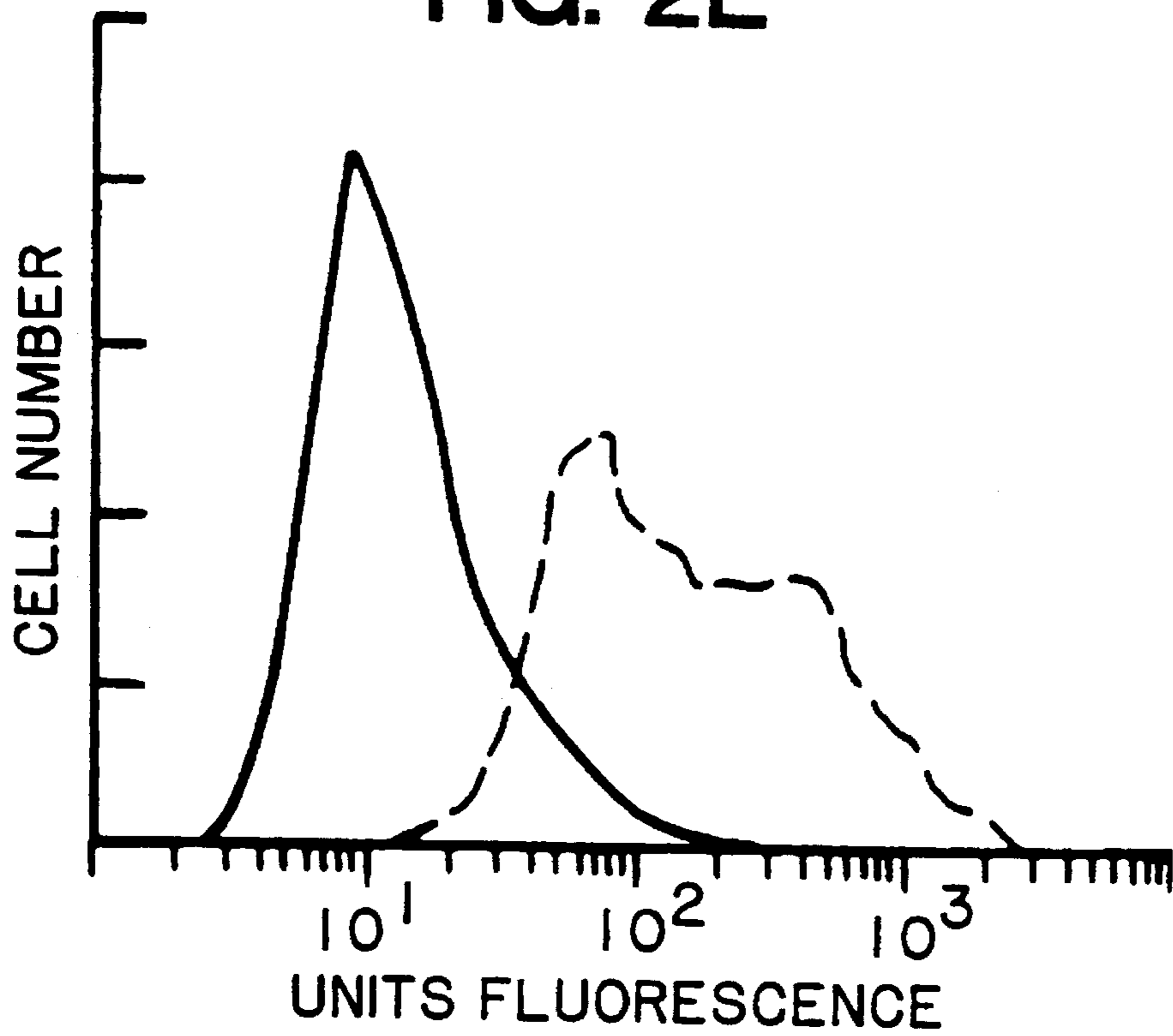


FIG. 2F

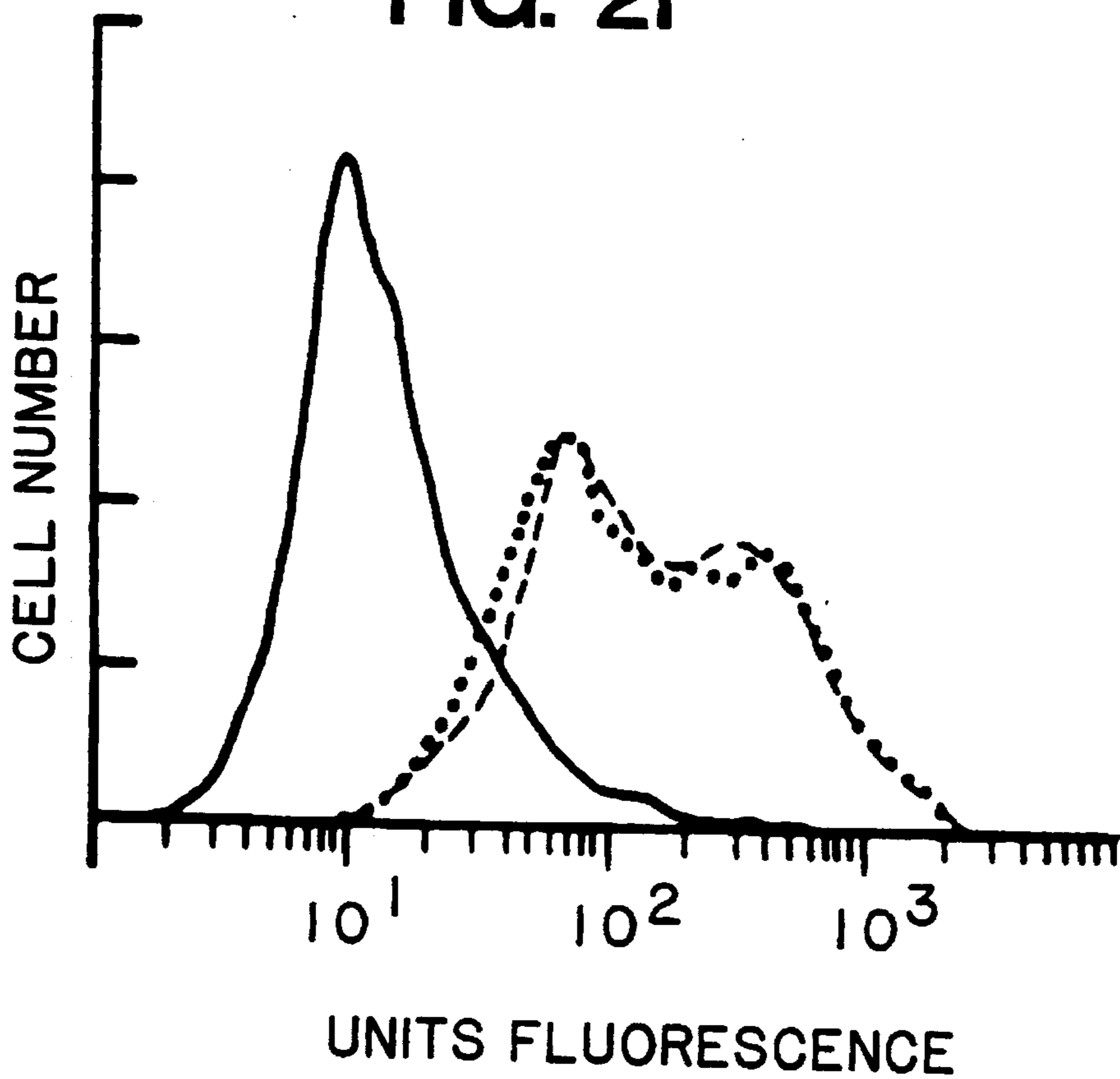


FIG. 2G

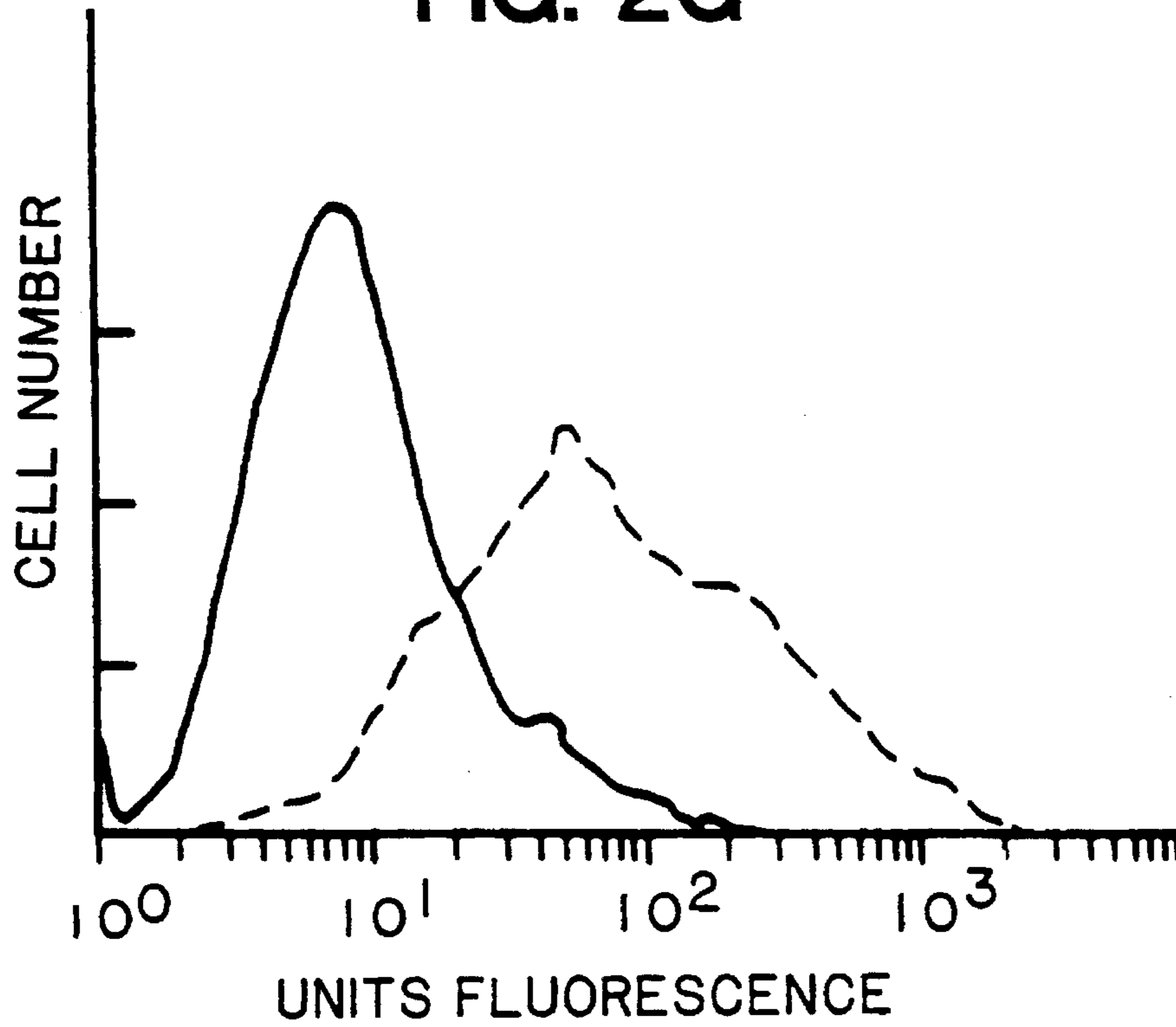


FIG. 2H

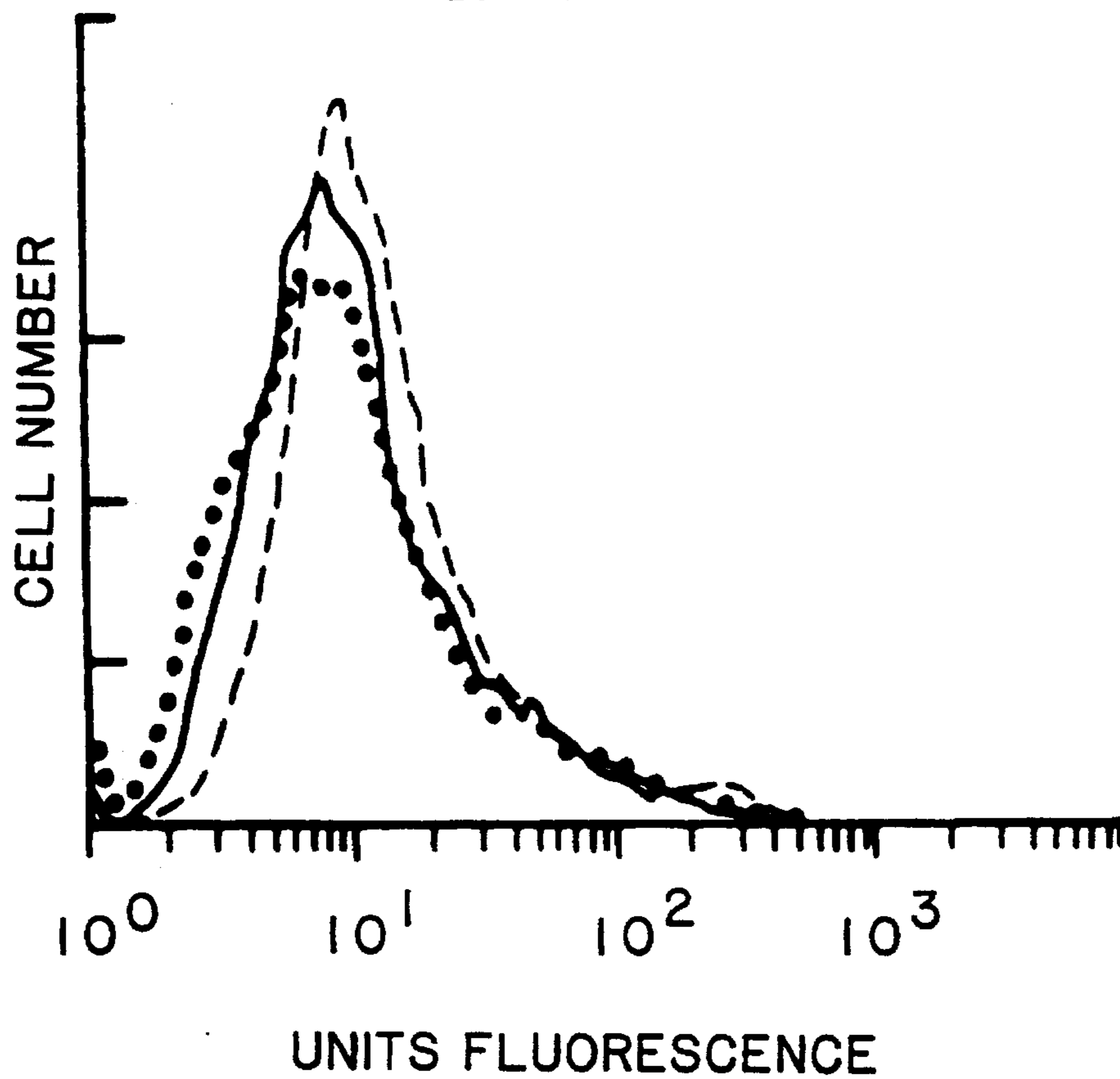


FIG. 2I

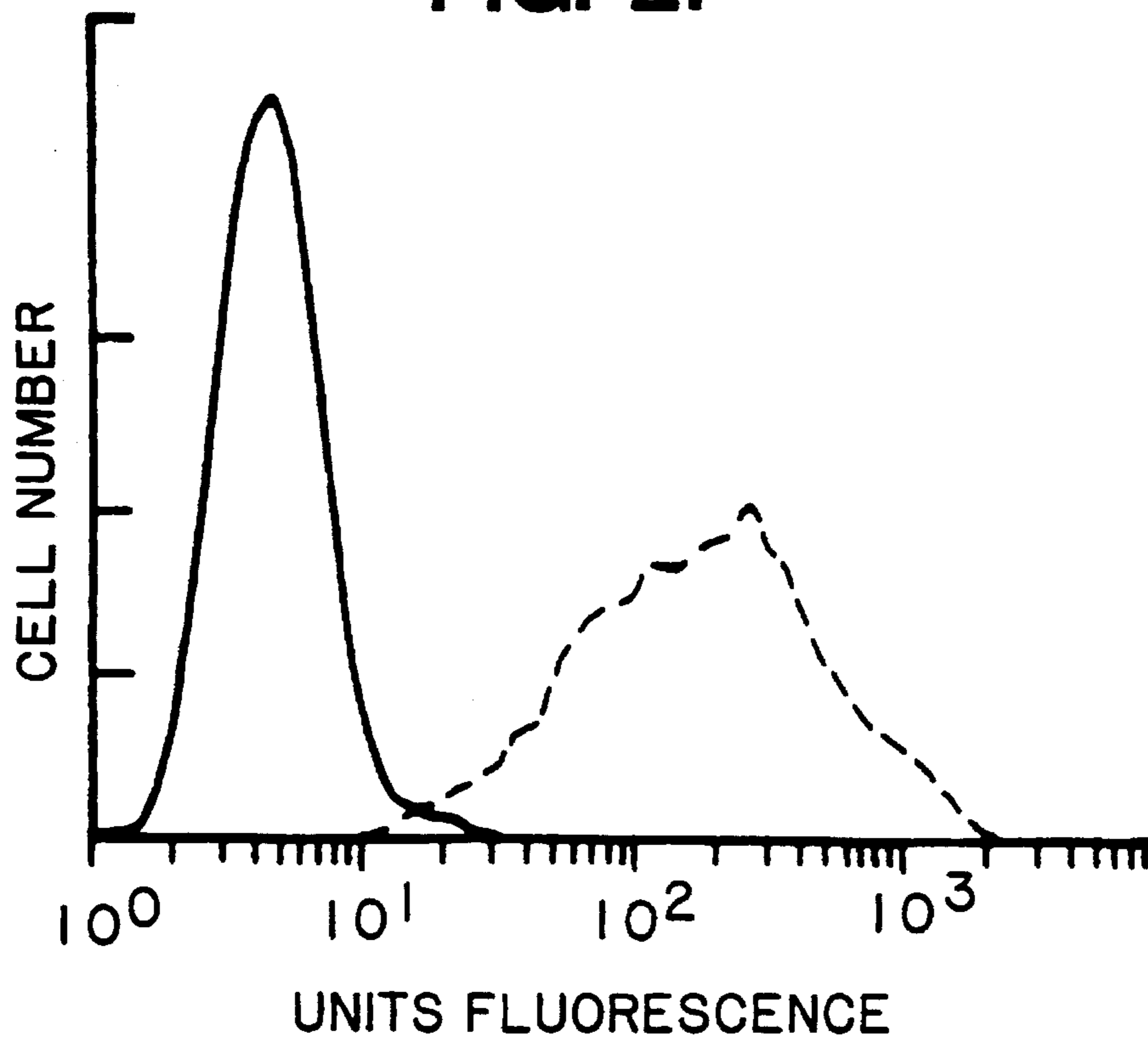


FIG. 2J

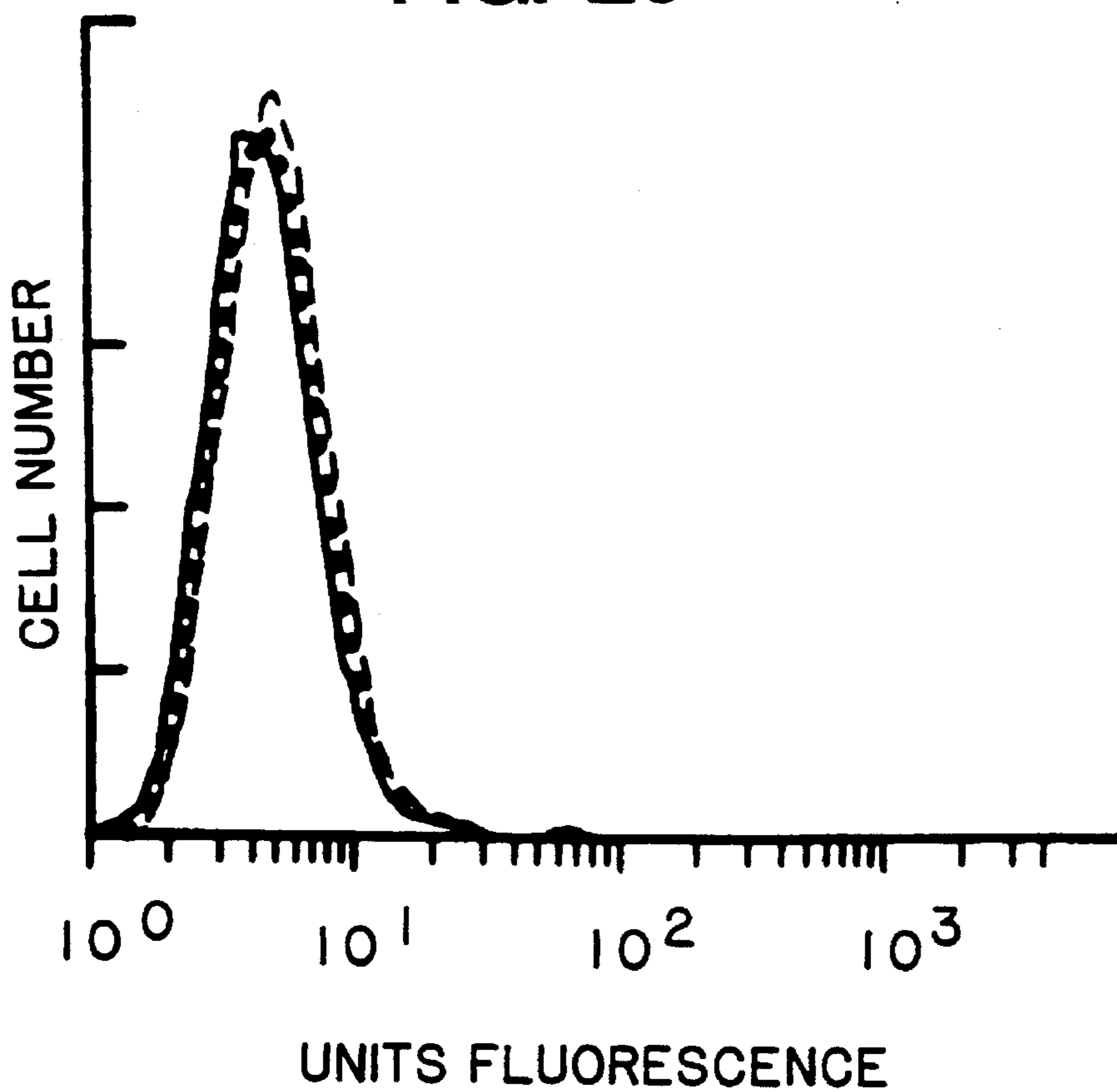


FIG. 2K

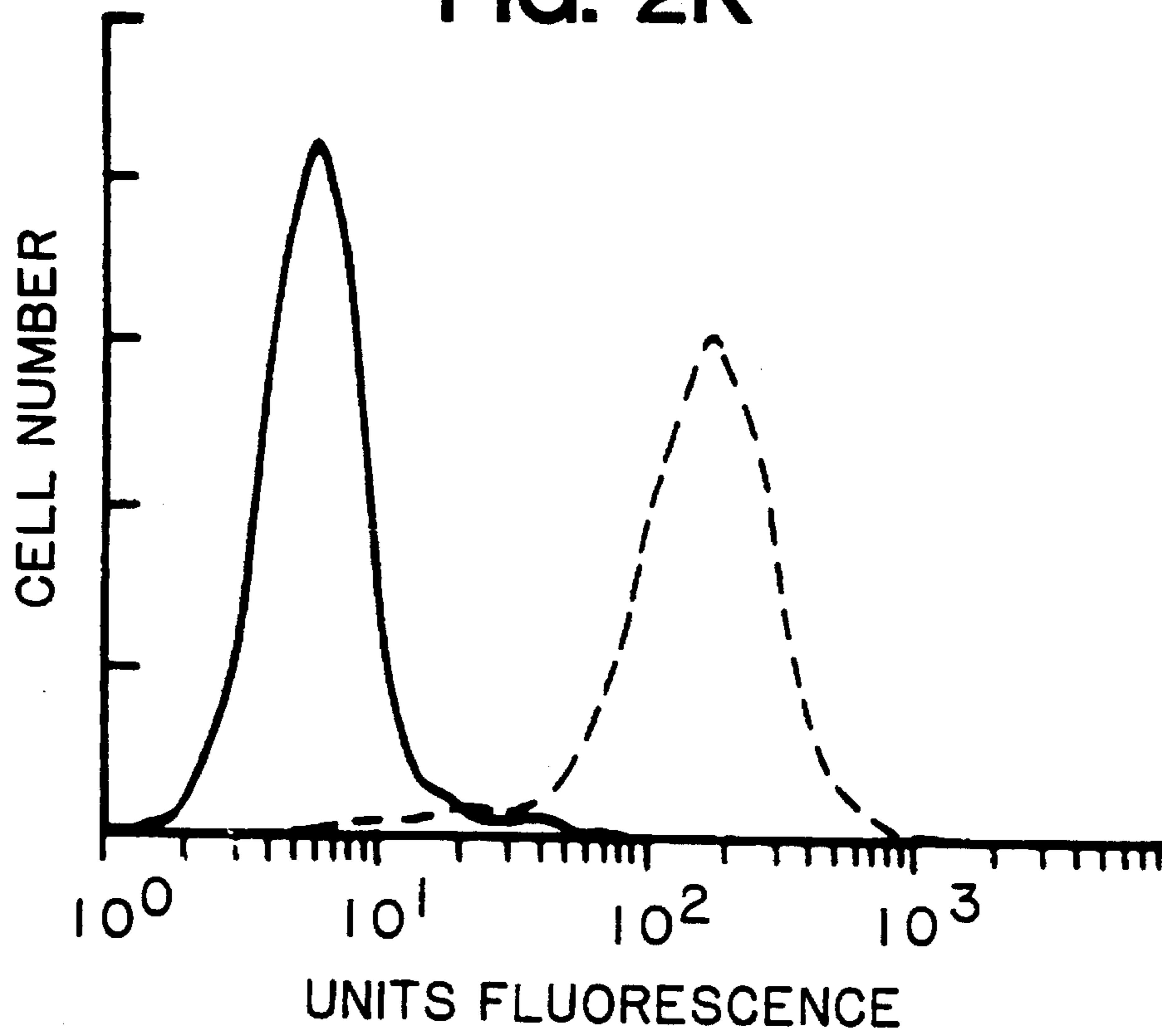


FIG. 2L

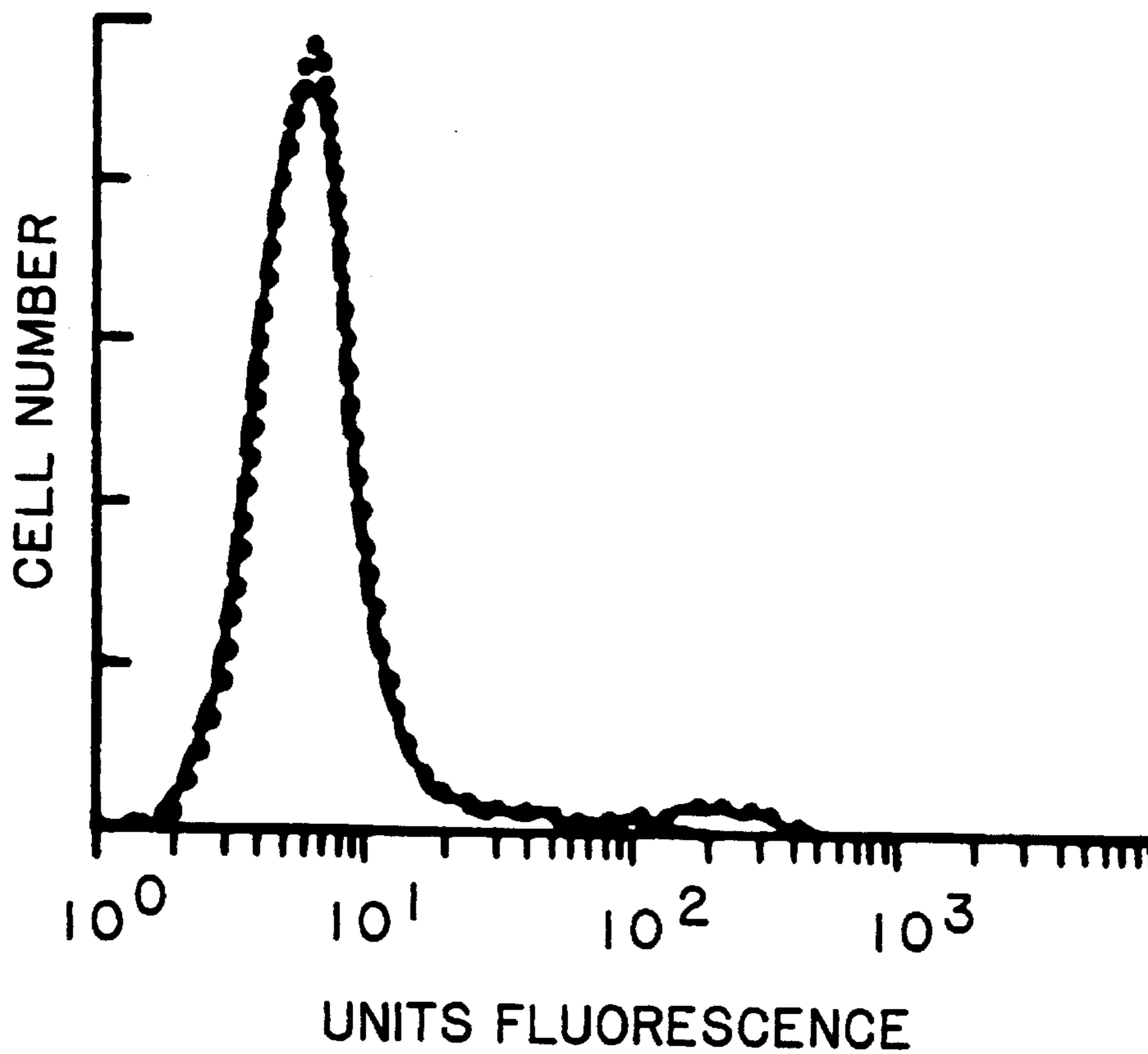


FIG. 2M

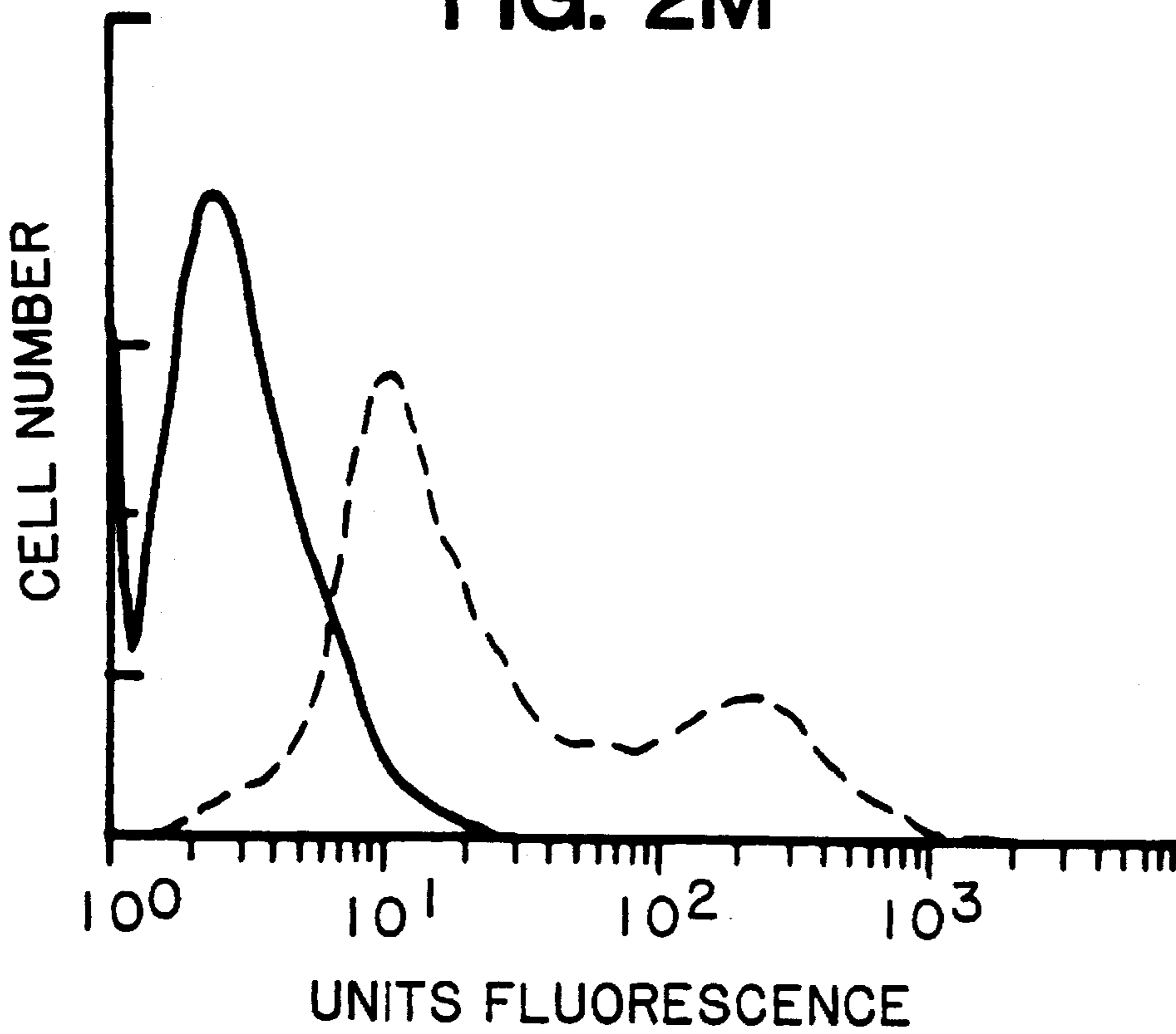


FIG. 2N

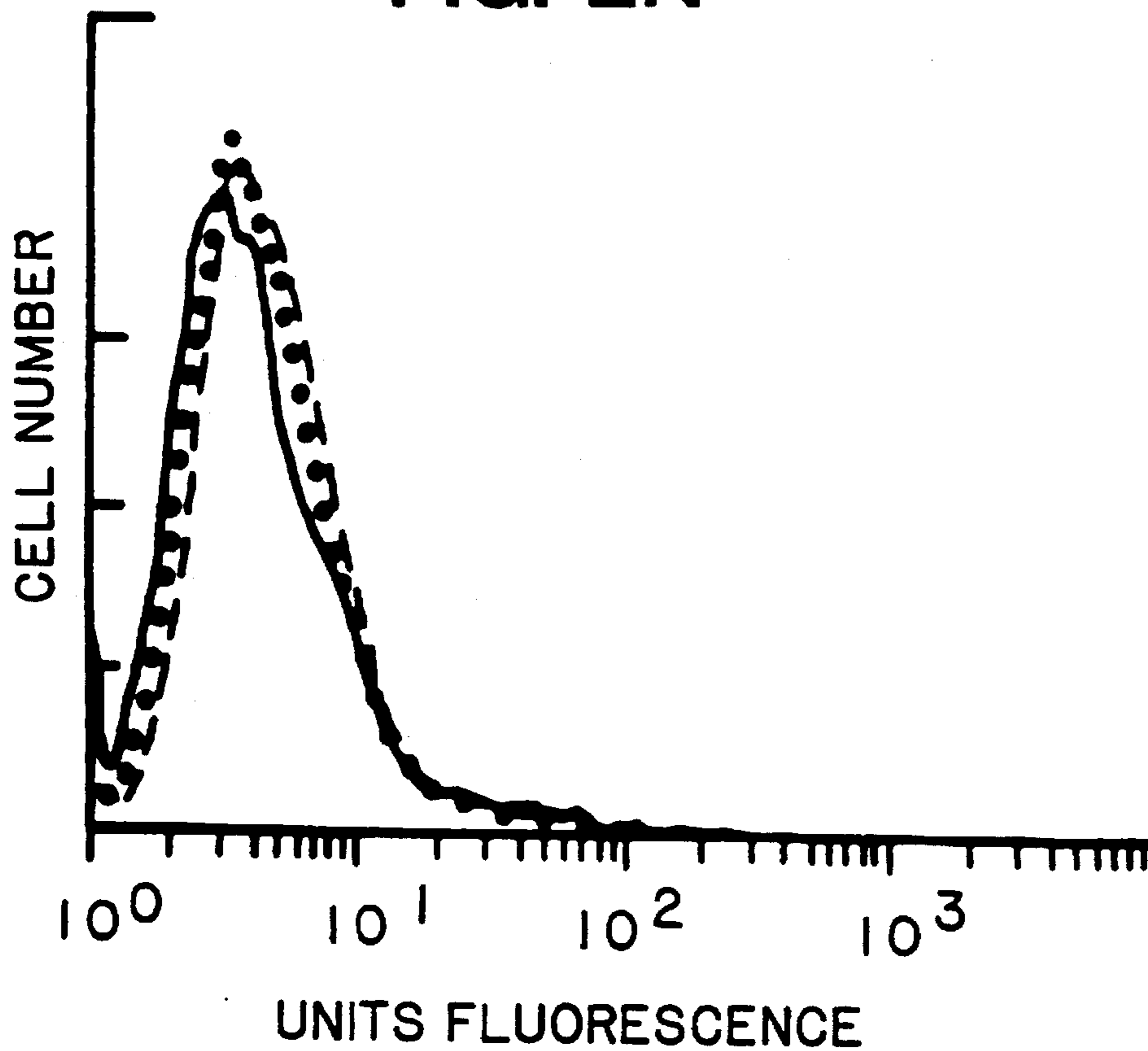


FIG. 3A

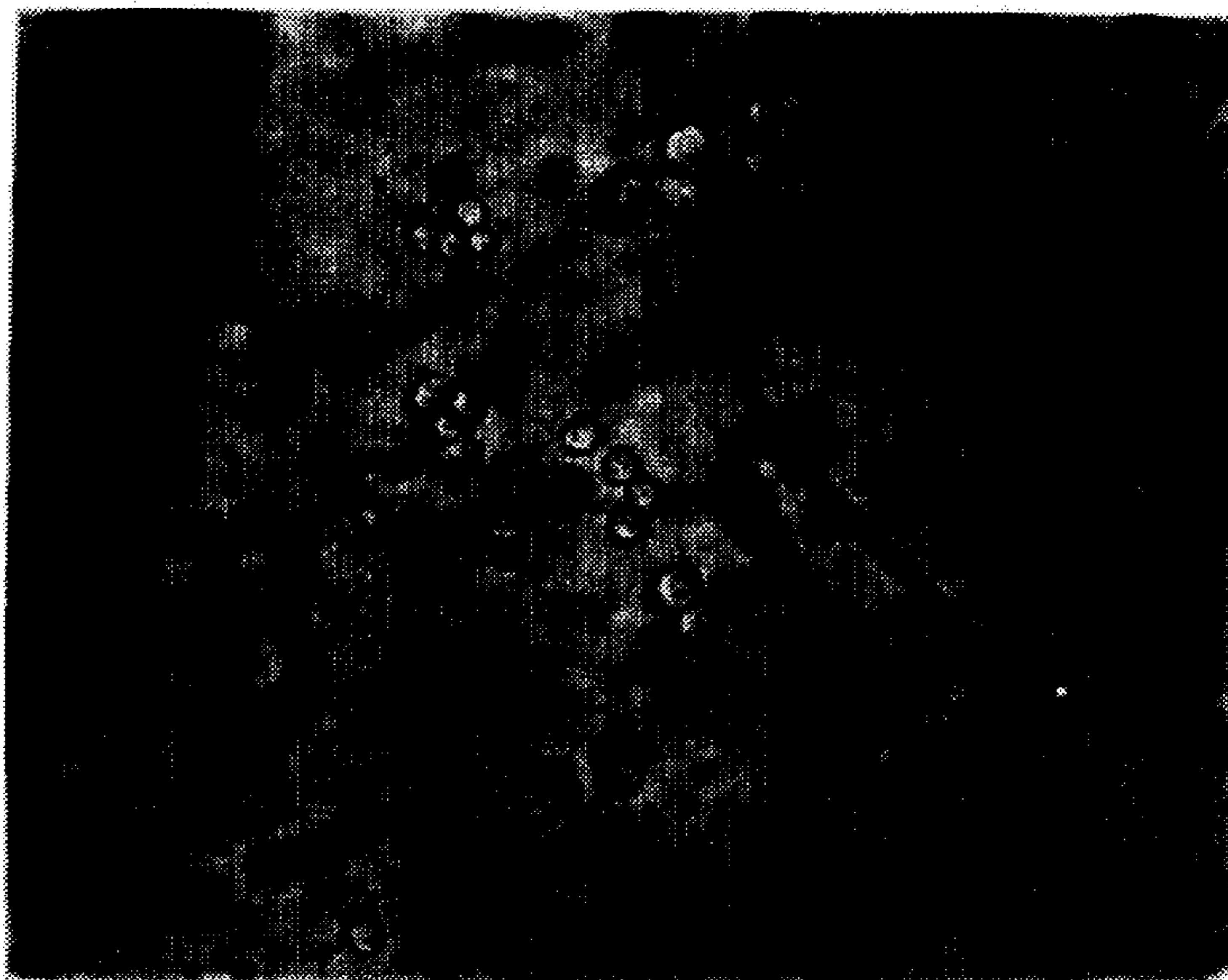


FIG. 3B

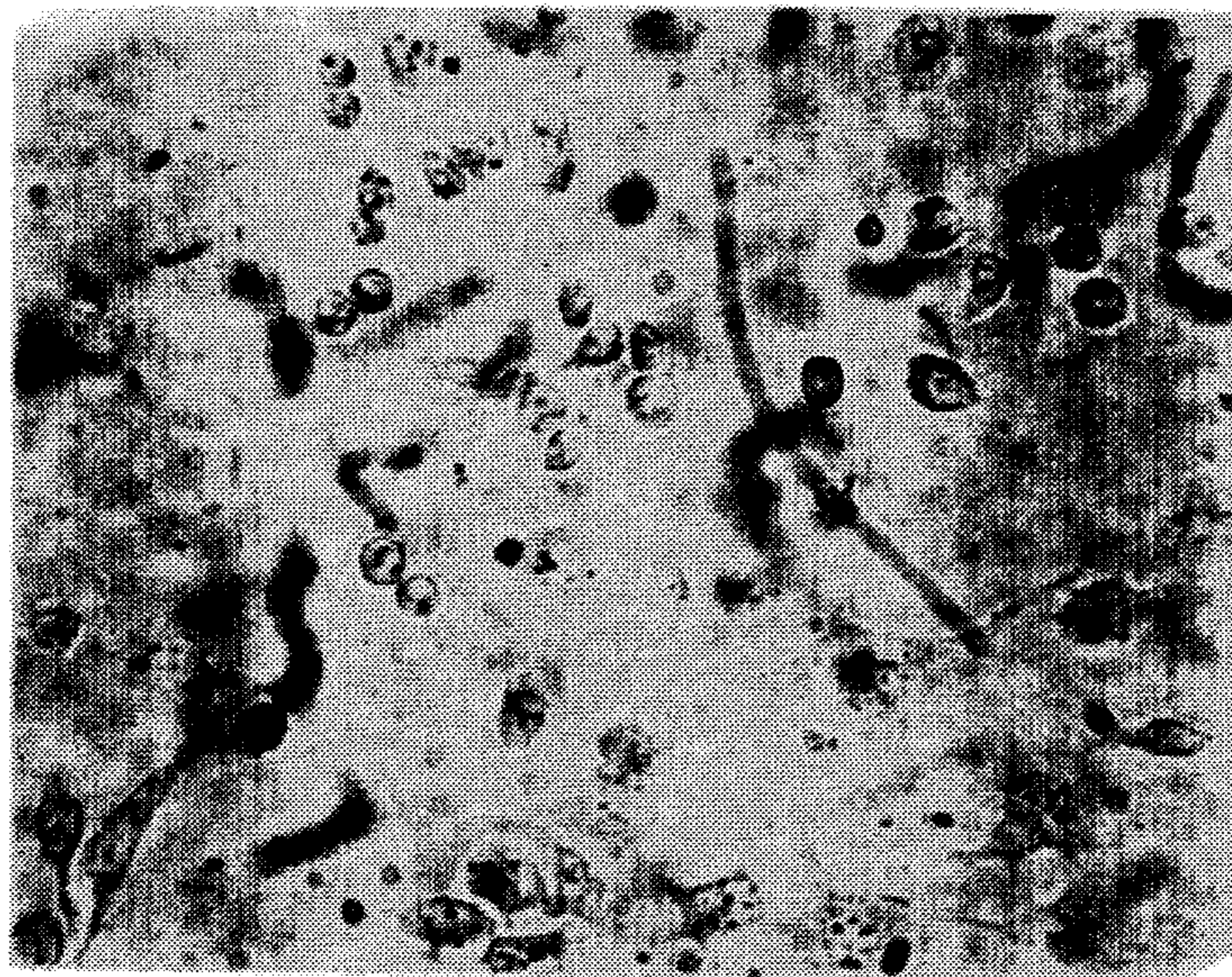


FIG. 3C

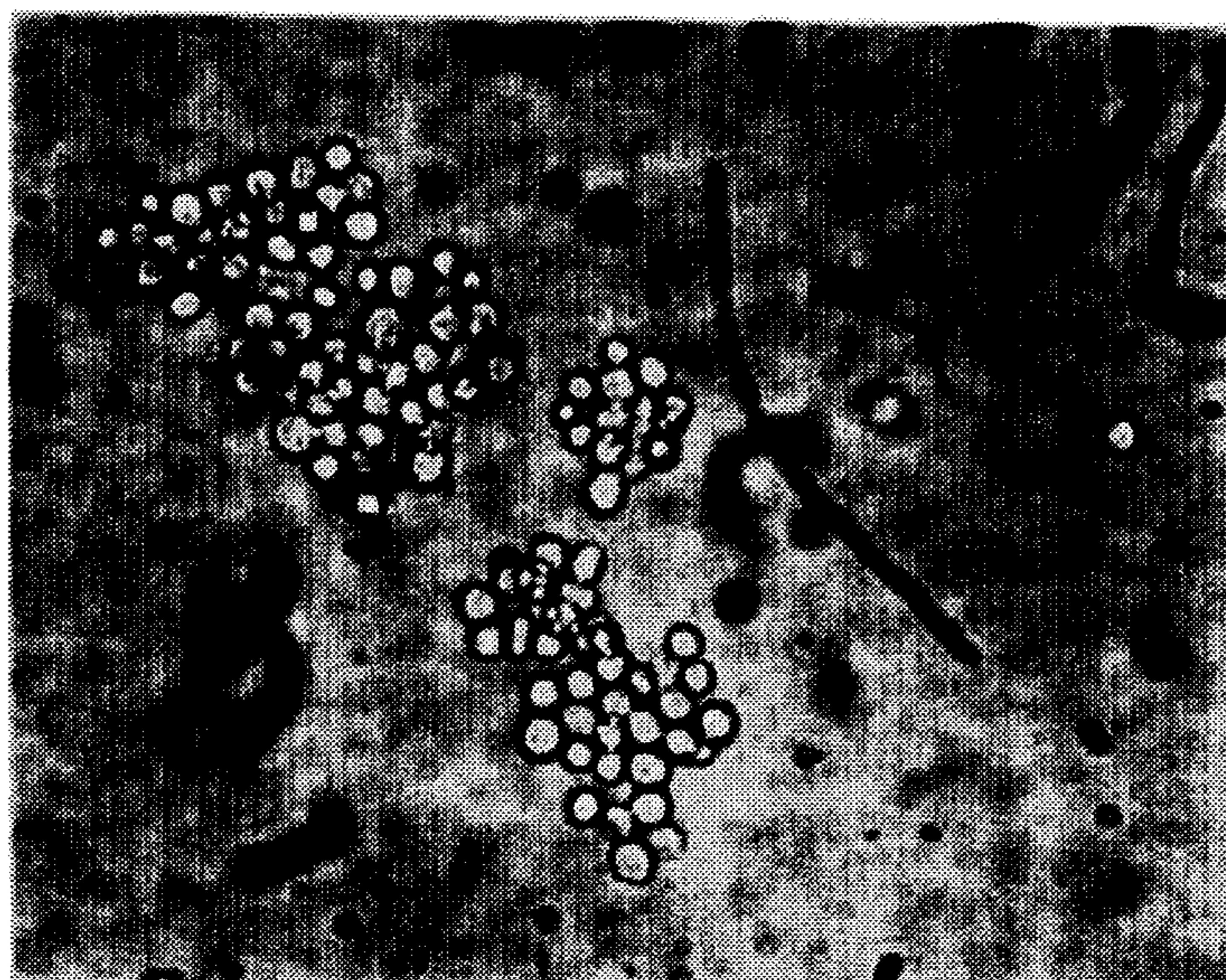


FIG. 4

CHO

M57

M16,3

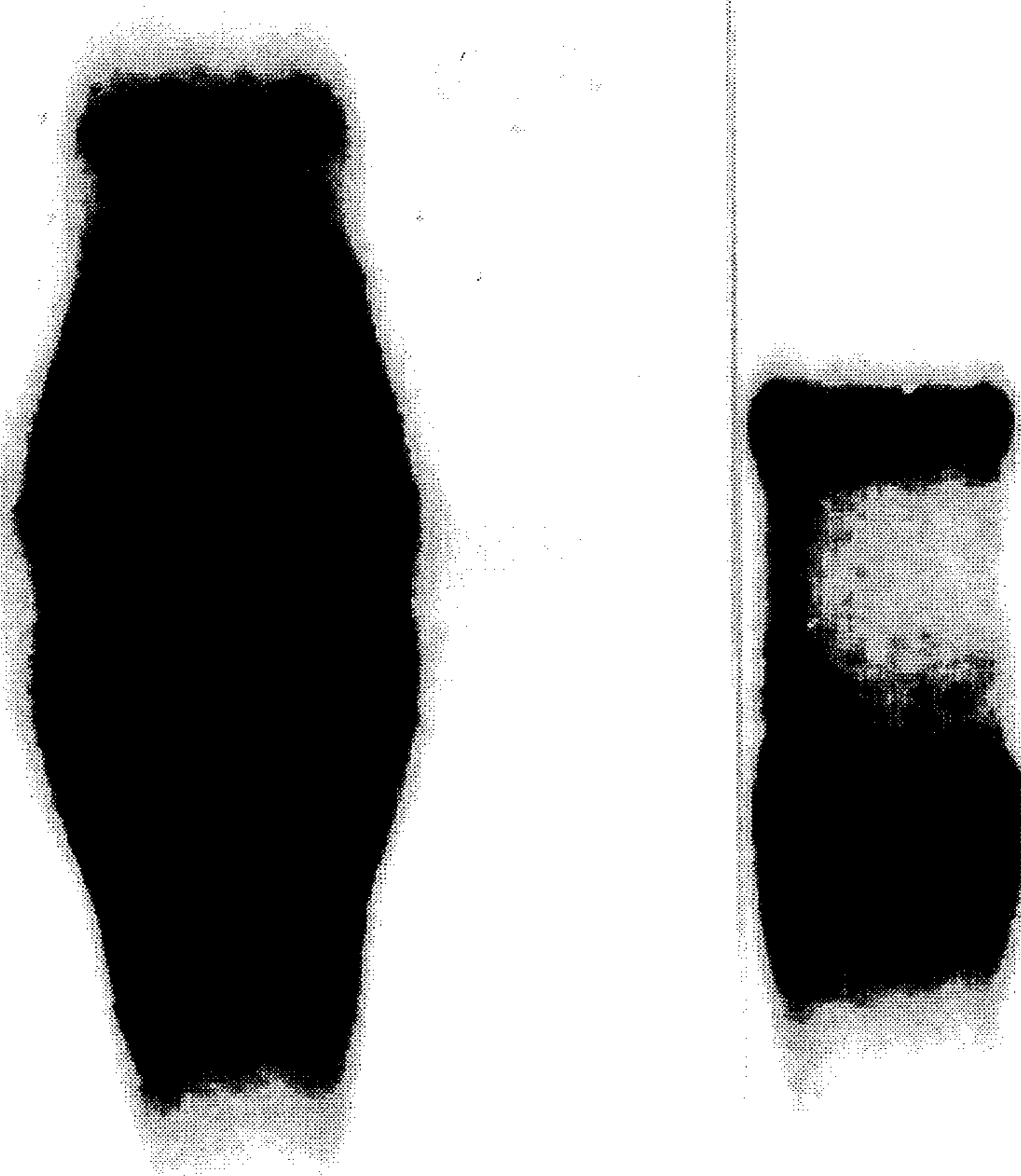


FIG. 5

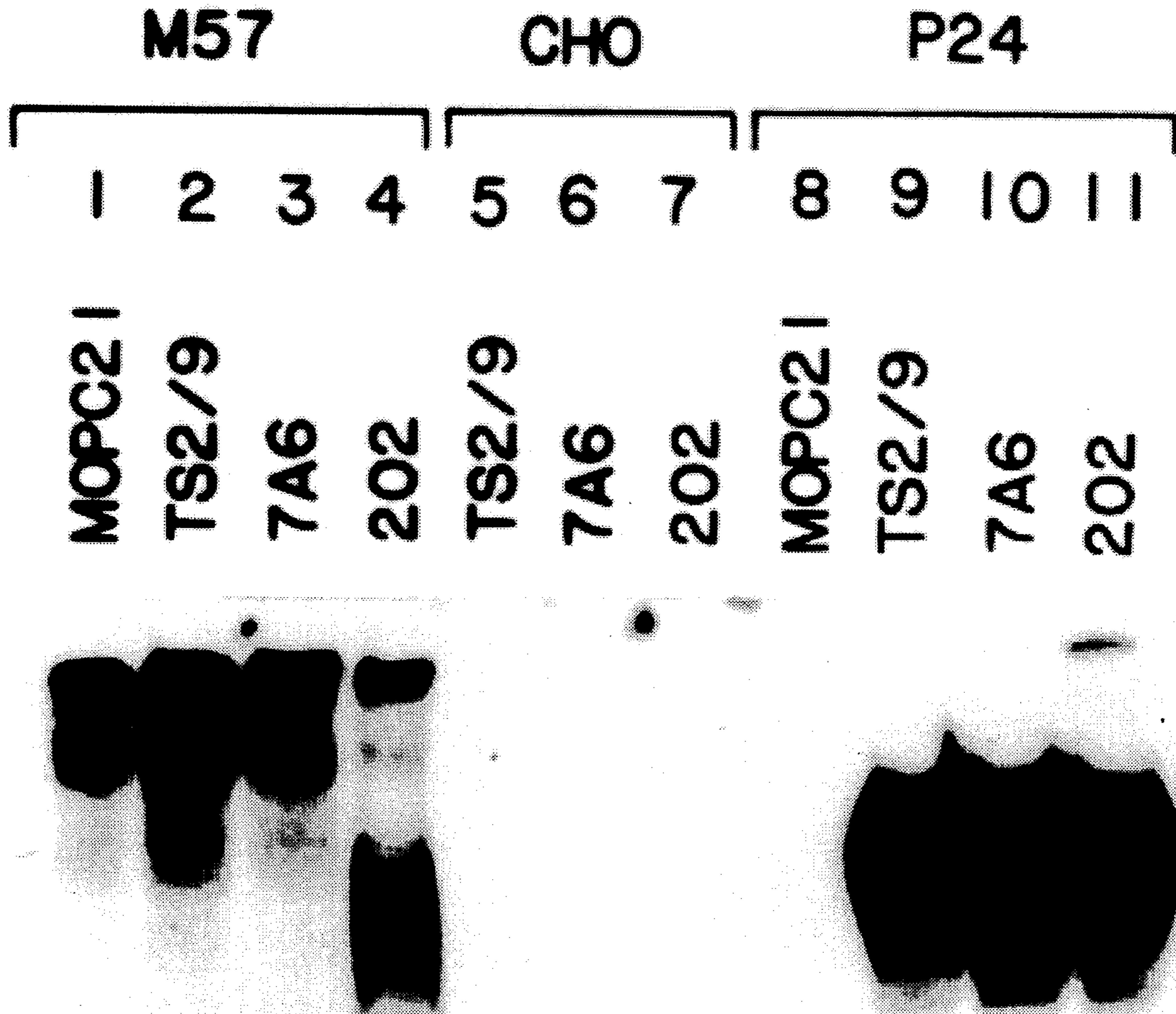


FIG. 6A-1

1 CGACGAGCCATGGTTGCTGGAGCGACGGCGGGCCCTGGGGTCCCT 50
 MetValAlaGlySerAspAlaGlyArgAlaLeuGlyValLe
 -28
 51 CAGCGTGGTCTGCCCTGCTGCACTGCTTTGGTTTCATCAGCTGTTTCCC 100
 uSerValValCysLeuLeuHisCysPheGlyPheIleSerCysPheSerG
 +1
 101 AACAAATATATGGTGTGTATGGGAATGTAACCTTCCATGTACCAAGC 150
 InGlnIleTyrGlyValValTyrGlyAsnValThrPheHisValProSer 19
 151 AATGTGCCCTTTAAAGAGGTCCTATGGAA[•]AAACAAAGGATAAAGTTGC 200
AsnValProLeuLysGluValLeuTrpLysLysGlnLysAspLysValAl 36
 201 AGAACTGGAA[•]AATTCGAA[•]ATCAGAGCTTTCATCTTTTAA[•]AAATAGGG 250
aGluLeuGluAsnSerGluPheArgAlaPheSerSerPheLysAsnArgV 53
 M 100
 251 TTTATTTAGACACTGTGTCAGGTAGCCTCACTATCTACA[•]ACTTAACATCA 300
aITyrLeuAspThrValSerGlySerLeuThrIleTyrAsnLeuThrSer 69

301 TCAGATGAAGATGAGTATGAAATGGAAATCGCCAATAATTACTGATACCAT 350
SerAspGluAspGluTyrGluMetGluSerProAsnIleThrAspThrMe 86

351 GAAGTCTTCTTATGTGCTTGAGTCTCTCCATCTCCACACTAACTT 400
tLysPheLeuTyrValLeuGluSerLeuProSerProThrLeuThrC 103
 M 101

401 GTGCATTGACTAATGGAAGCATTGAAGTCCAATGCATGATACCAGAGCAT 450
ySAlaLeuThrAsnGlySerIleGluValGlnCysMetIleProGluHis 119

451 TACAACAGCCATCGAGGACTTATAATGTACTCATGGGATTGTCCCTATGGA 500
TyrAsnSerHisArgGlyLeuIleMetTyrSerThrPaspCysPrometGI 136

501 GCAATGTAACGTAACCTCAACCAGTATAATTTAAGATGGAAAATGATC 550
uGlnCysLysArgAsnSerThrSerIleTyrPheLysMetGluAsnAspL 153
 M 102

FIG. 6A-2

FIG. 6B

551	TTCACAAAATACAGTCTTAGCAATCCATTATTAAATACAACA <u>euPROGlnLysIleGlnCysThrLeuSerAsnProLeuPheAsnThrThr</u>	600 169
601	TCATCAATCATTTTGACAACCTGTATCCCAAGCAGCGGTCATTCAGACA <u>SerSerIleIleLeuThrThrCysIleProSerSerGlyHisSerArgHi</u>	650 186
651	CAGATATGCACCTTATACCCATACCATTAGCAGTAATTACAACATGTATTG sArgTyrAlaLeuIleProIleProLeuAlaValIleThrThrCysIleV	700 203
701	TGCTGTATATGAATGGTATTCTGAAATGTGACAGAAAACCAGACAGAACC alLeuTyrMetAsnGlyIleLeuLysCysAspArgLysProAspArgThr	750 219
751	AACTCCAATTGATTGGTAACAGAGATGAAGACAACAGCATAACTAAATT AsnSerAsn	800 222
801	ATTTTAAAACCTAAAAGCCATCTGATTTCTCATTTGAGTATTACAATTT	850
851	TTGAACAACCTGTTGGAAATGTAAGTGAAGCAGCTGCTTTAAGAAGAAAT	900
901	ACCCACTAACAAAGAACAAGCATTAGTTTGGCTGTCATCAACTTATTAT	950
951	ATGACTAGGTGCTTGCTTTTTTTTGTGCAGTAAATGTTTTTACTGATGATG	1000
1001	TAGATACTTTTGTAAATAAATGTAATAATATGTACACAAGTG	1040

FIG. 7A



FIG. 7B

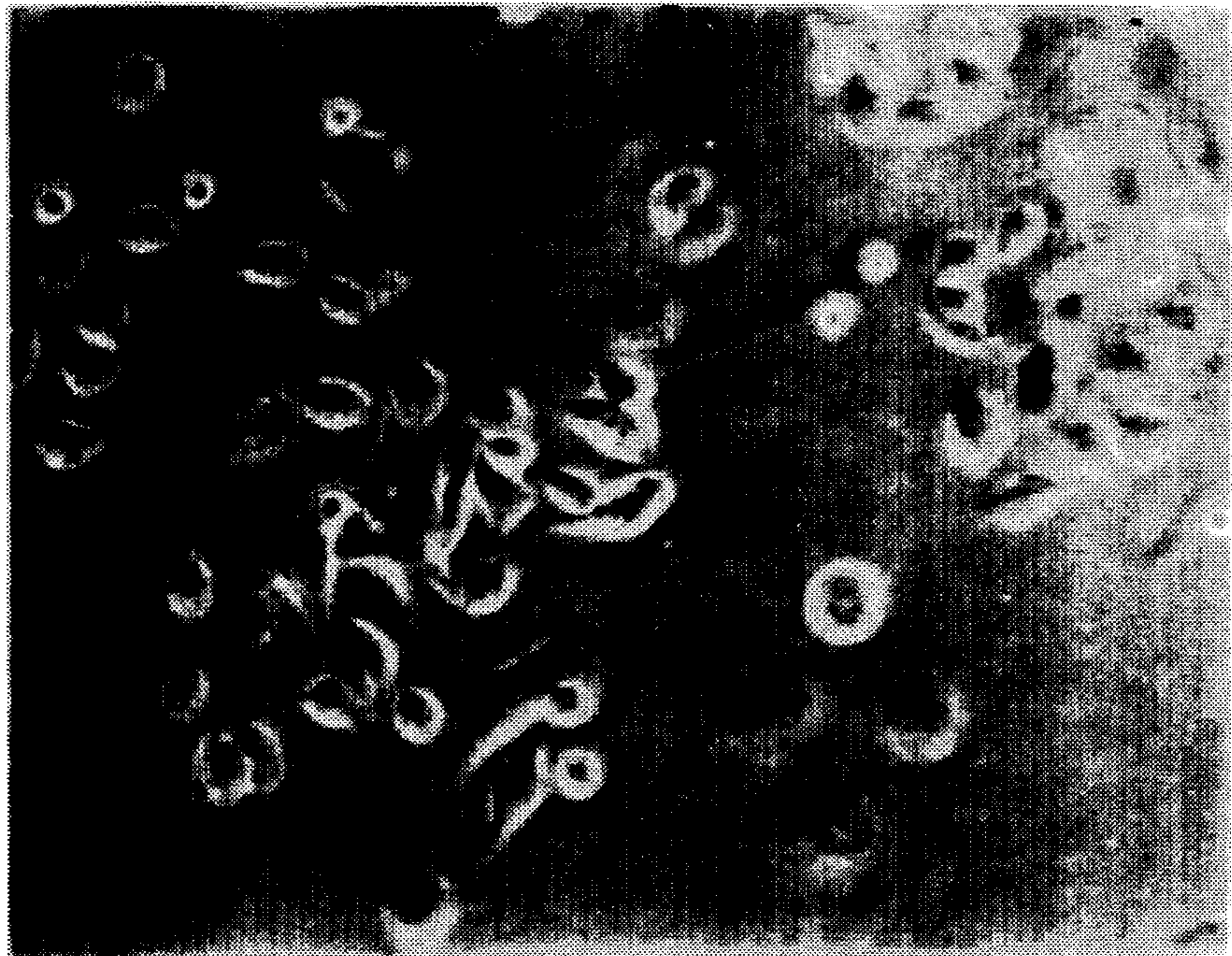


FIG. 7C

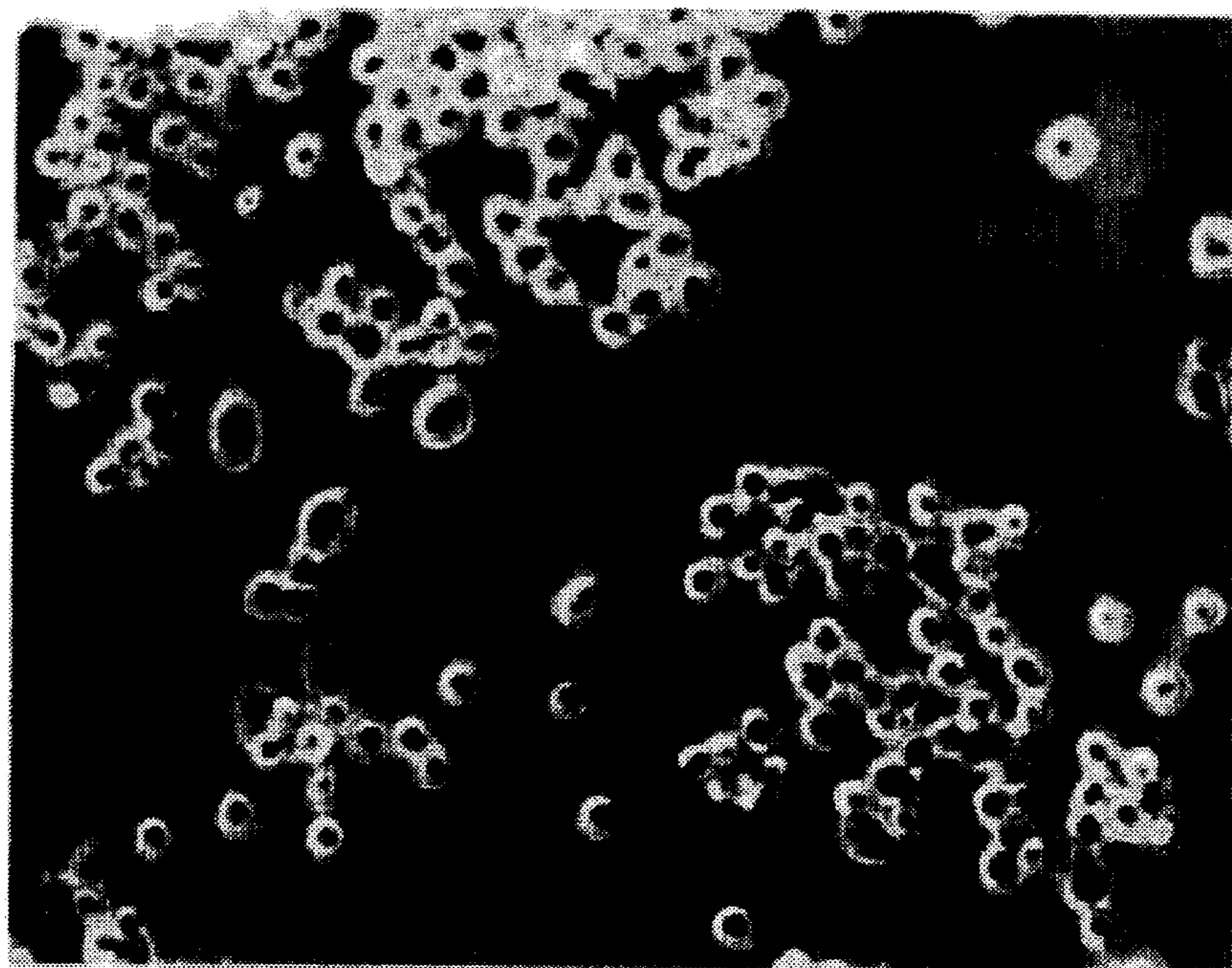


FIG. 7D

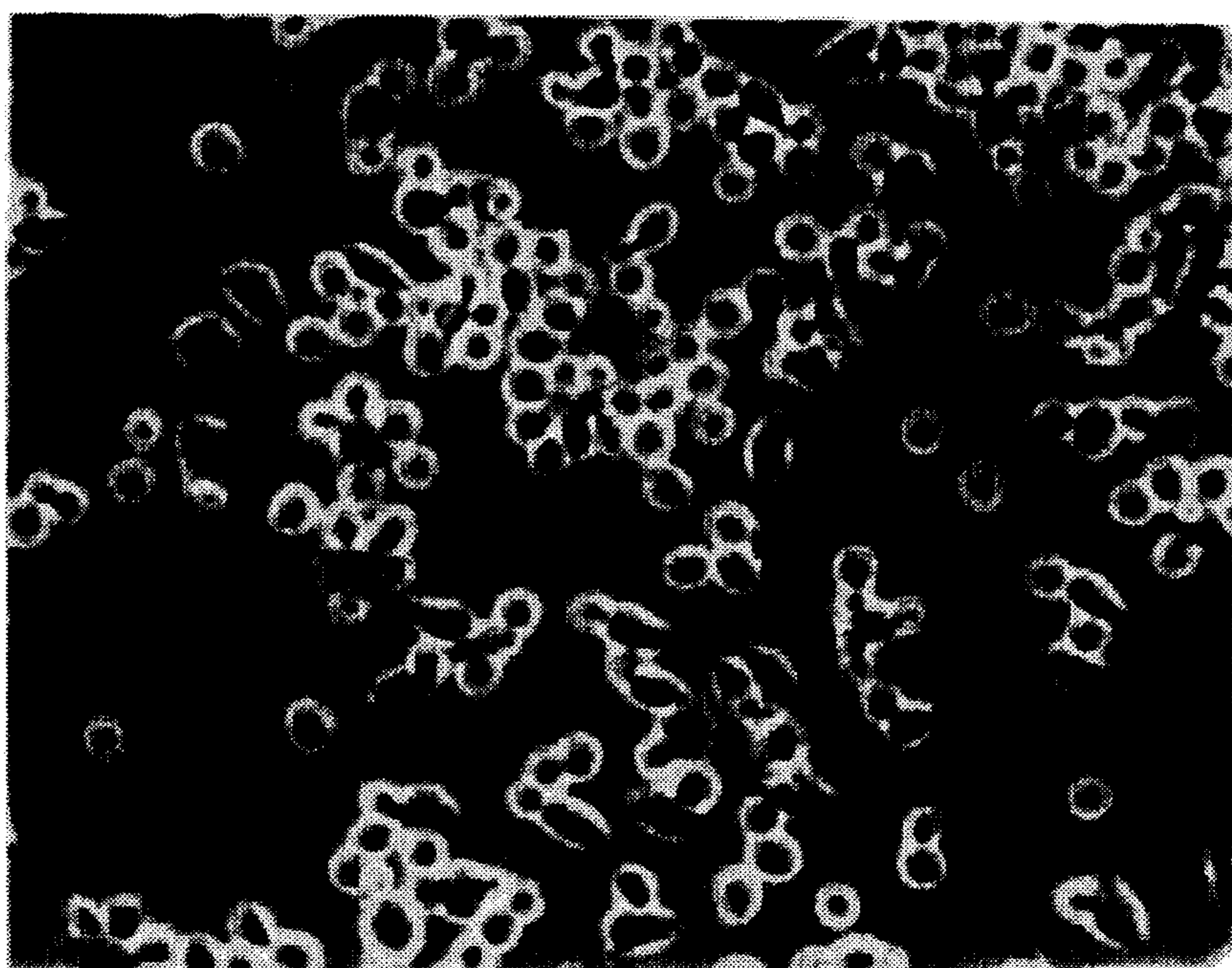


FIG. 8A

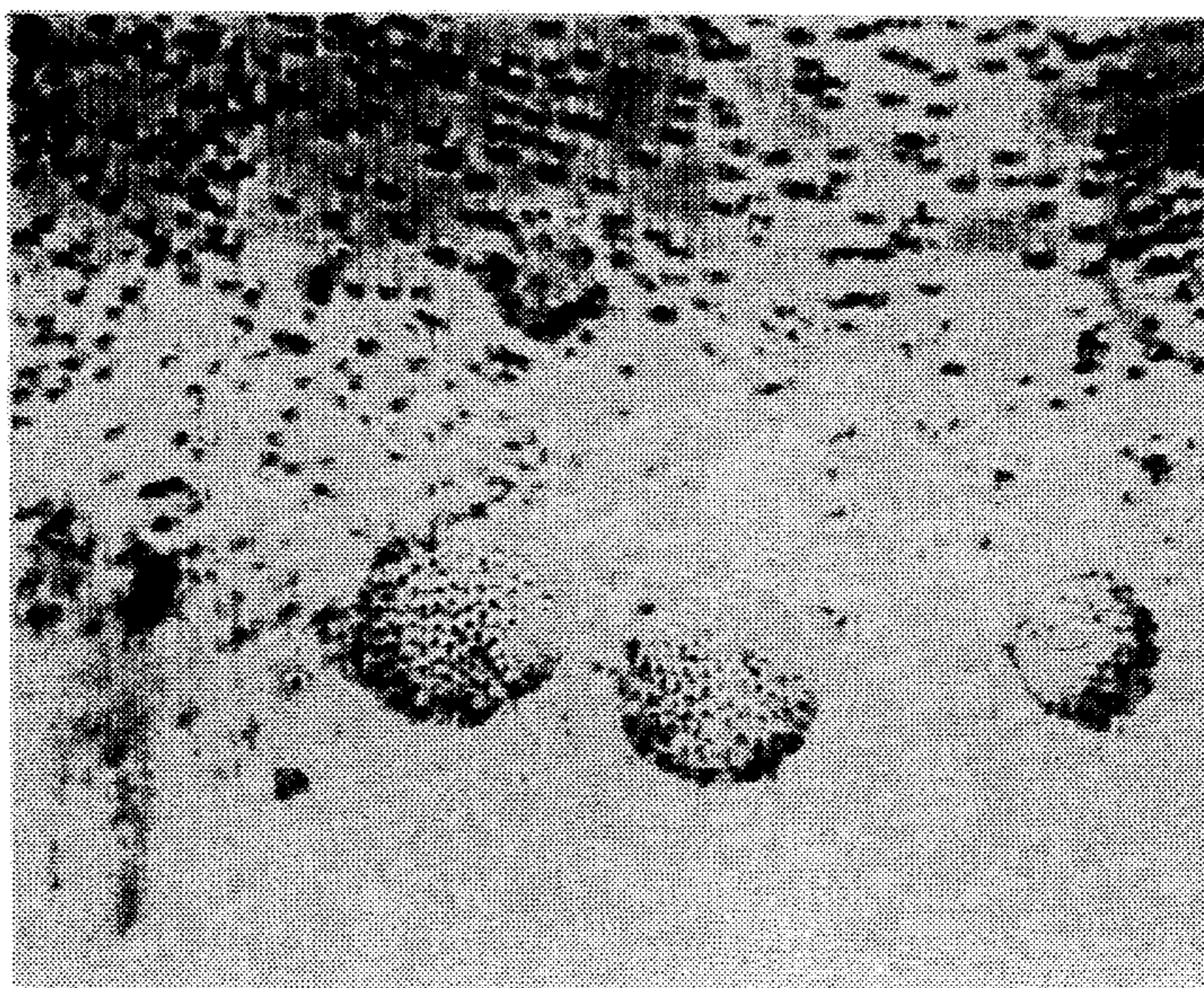


FIG. 8B

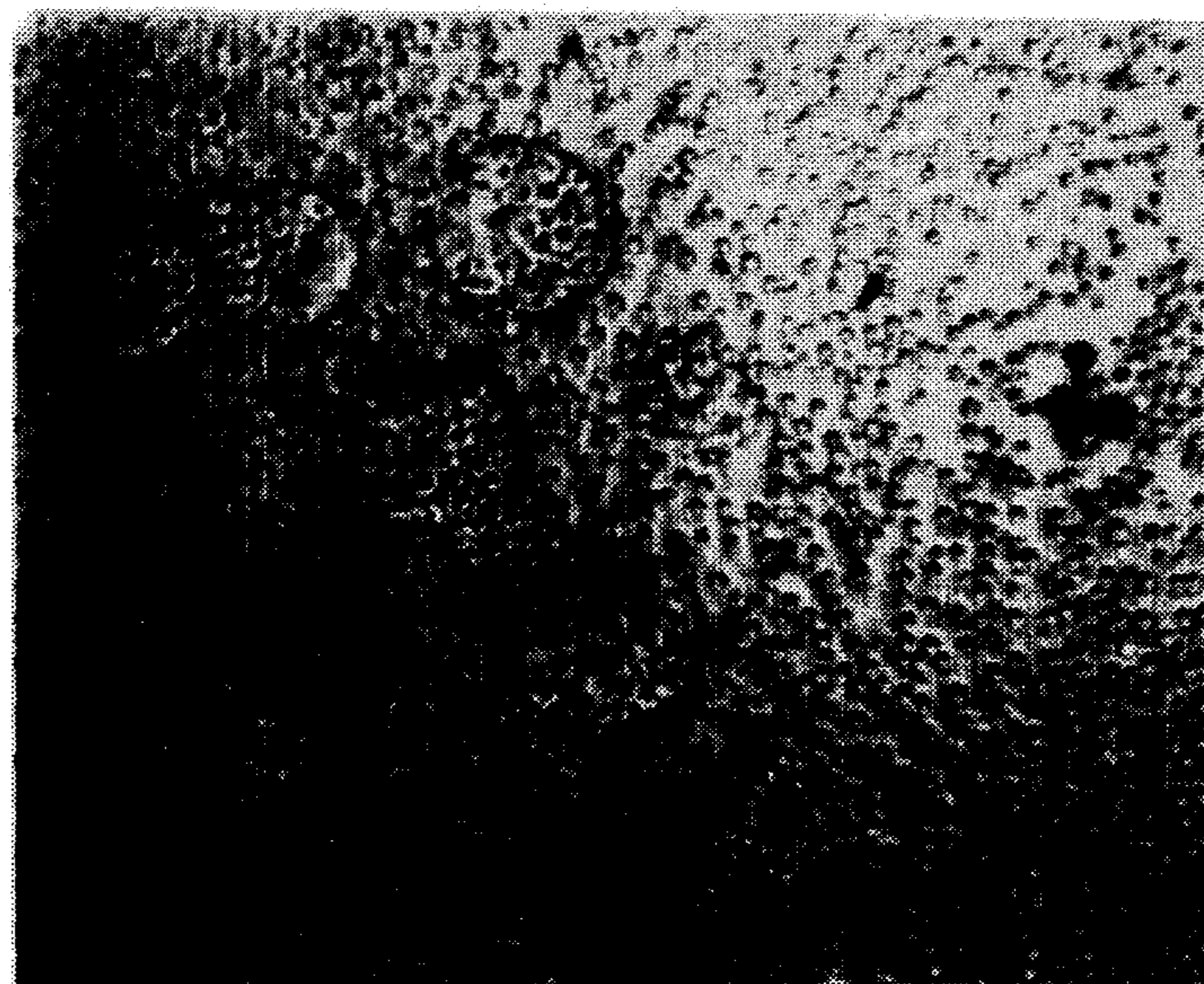


FIG. 8C

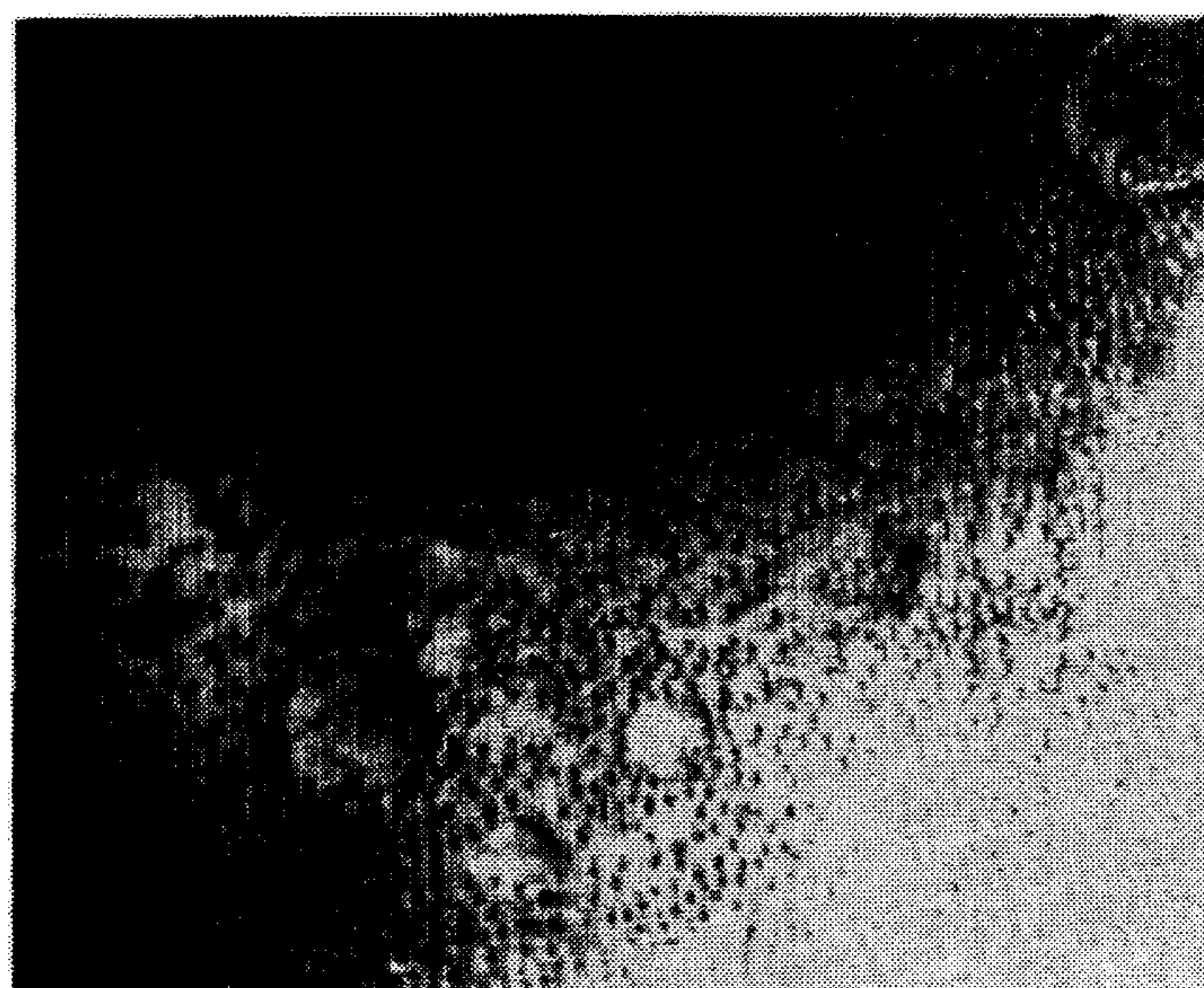


FIG. 9A

1	GCGGCCCGACGAGCCATGGTTGCTGGAGCGACGCGGGCCCTG	50
	MetValAlaGlySerAspAlaGlyArgAlaLeu	
51	GGGTCCTCAGCGTGGTCTGCCCTGCTGCACCTGCTTGGTTTCATCAGCTG	100
	GlyValLeuSerValValCysLeuLeuHisCysPheGlyPheIleSerCys	
101	TTTTTCCCAACAATAATATGGTGTGTATGGGAATGTAACCTTCCATG	150
	sPheSerGlnGlnIleTyrGlyValValTyrGlyAsnValThrPheHisV	
	+1	
151	TACCAAGCAATGTGCCCTTTAAAGAGGTCCTATGGAAAACAAGGAT	200
	alProSerAsnValProLeuLysGluValLeuTrpLysLysGlnLysAsp	
201	AAAGTTGCAGAACTGGAAAATTCTGAATTCAGAGCTTTCATCTTTAA	250
	LysValAlaGluLeuGluAsnSerGluPheArgAlaPheSerSerPheLys	+50
251	AAATAGGGTTTATTAGACACTGTGTCAGGTAGCCTCACTATCTACAAC	300
	sAsnArgValTyrLeuAspThrValSerGlySerLeuThrIleTyrAsnL	
301	TAACATCATCAGATGAAGATGAGTATGAAATGGAATCGCCAAATATTACT	350
	euThrSerSerAspGluAspGluTyrGluMetGluSerProAsnIleThr	
351	GATACCATGAGTCTTCTTTATGTGCTTGAGTCTTCCATCTCCAC	400
	AspThrMetLysPhePheLeuTyrValLeuGluSerLeuProSerProTh	+100

401 ACTAACTTGTCATTGACTAATGGAAGCAATGAAGTCCAATGCATGATAC 450
rLeuThrCysAlaLeuThrAsnGlySerIleGluValGlnCysMetIleP
 451 CAGAGCATTACAACAGCCATCGAGGACTTATAATGTAATGTAATGGAATTGT 500
rOgluHisTyrAsnSerHisArgGlyLeuIleMetTyrSerTrpAspCys
 501 CCTATGGAGCAATGTAACGTAACCTCAACCAGTATAATTTAAGATGGA 550
PrometGluGlnCysLysArgAsnSerThrSerIleTyrPheLysMetGlu
 551 AAATGATCTTCCACAAAATAACAGTGTACTCTTAGCAATCCATTATTTA 600
uAsnAspLeuProGlnLysIleGlnCysThrLeuSerAsnProLeuPheA
 601 ATACAACATCATCATTTTGACAACCTGTATCCCAAGCAGCGGTCAT 650
 snThrThrSerSerIleIleLeuThrThrCysIleProSerSerGlyHis
 651 TCAAGACACAGATATGCACTTATACCCATACCATTAGCAGTAATTACAAC 700
 SerArgHisArgTyrAlaLeuIleProIleProLeuAlaValIleThrTh
 701 ATGTATTGTGCTGTATATGAATGGTATGTATGCTTTTAAACAATAATAG 750
 rCysIleValLeuTyrMetAsnGlyMetTyrAlaPhe
 751 TTTGAAAACCTTGCAATTTGTTCCAAAGGTCAGAAAATAGTTTAAGGATGA 800
 801 AAATAAAGTTTGAAATTTTAGACATTTGAAAAAAAATAAAAAAAA 850
 851 AAAAAGCGGCCGC 863

FIG. 9B

FIG. 10A

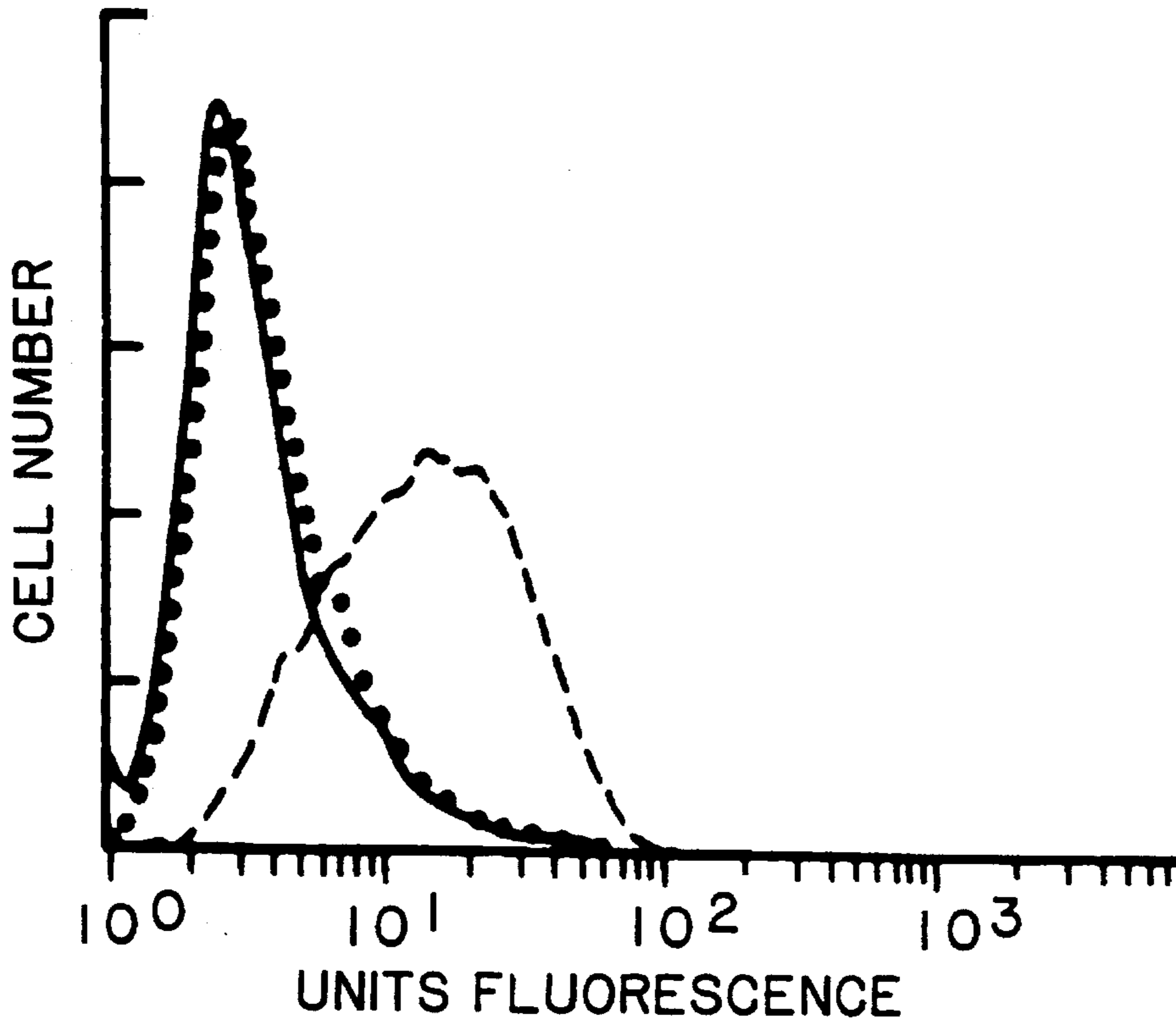


FIG. 10B

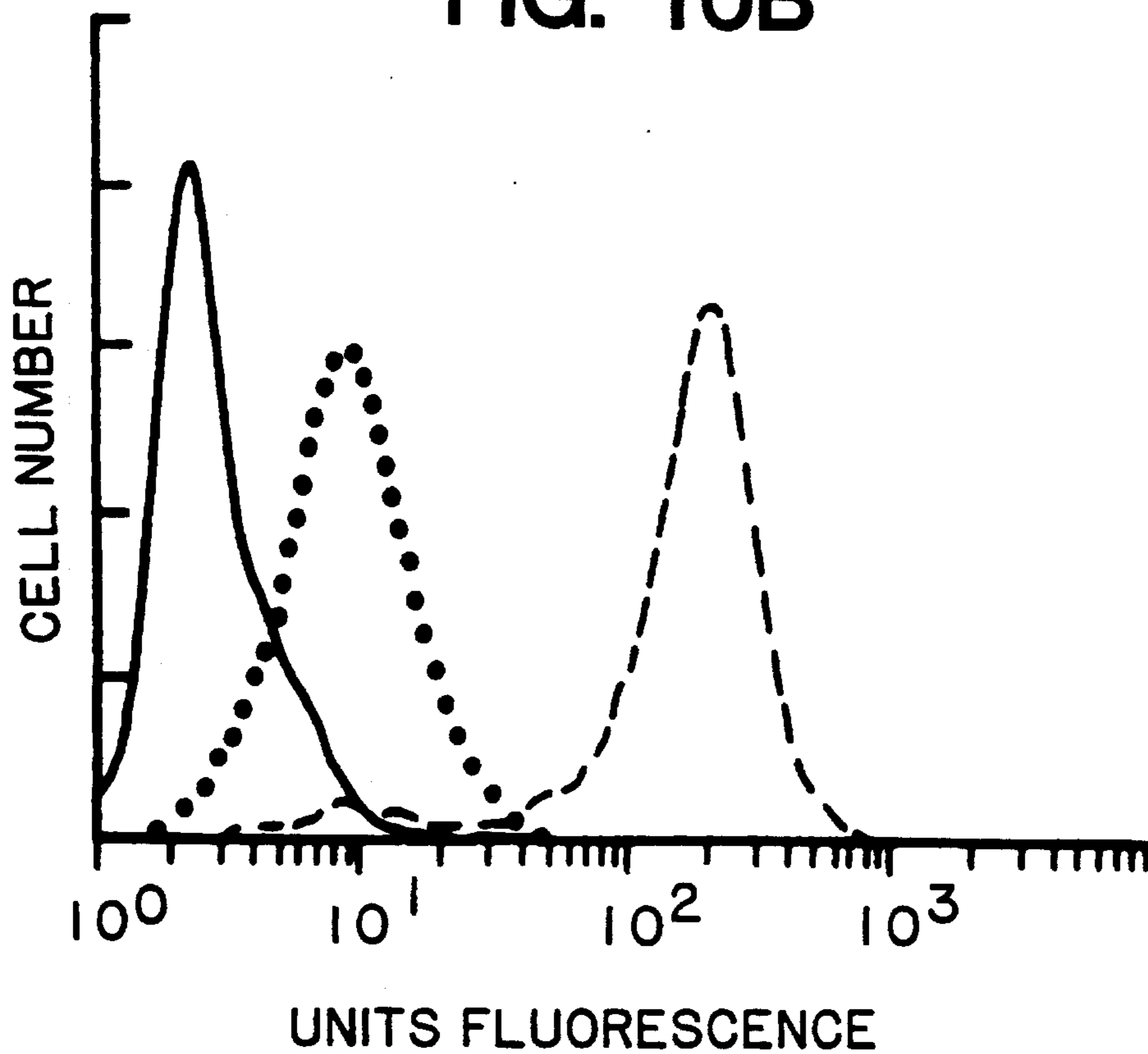


FIG. 11

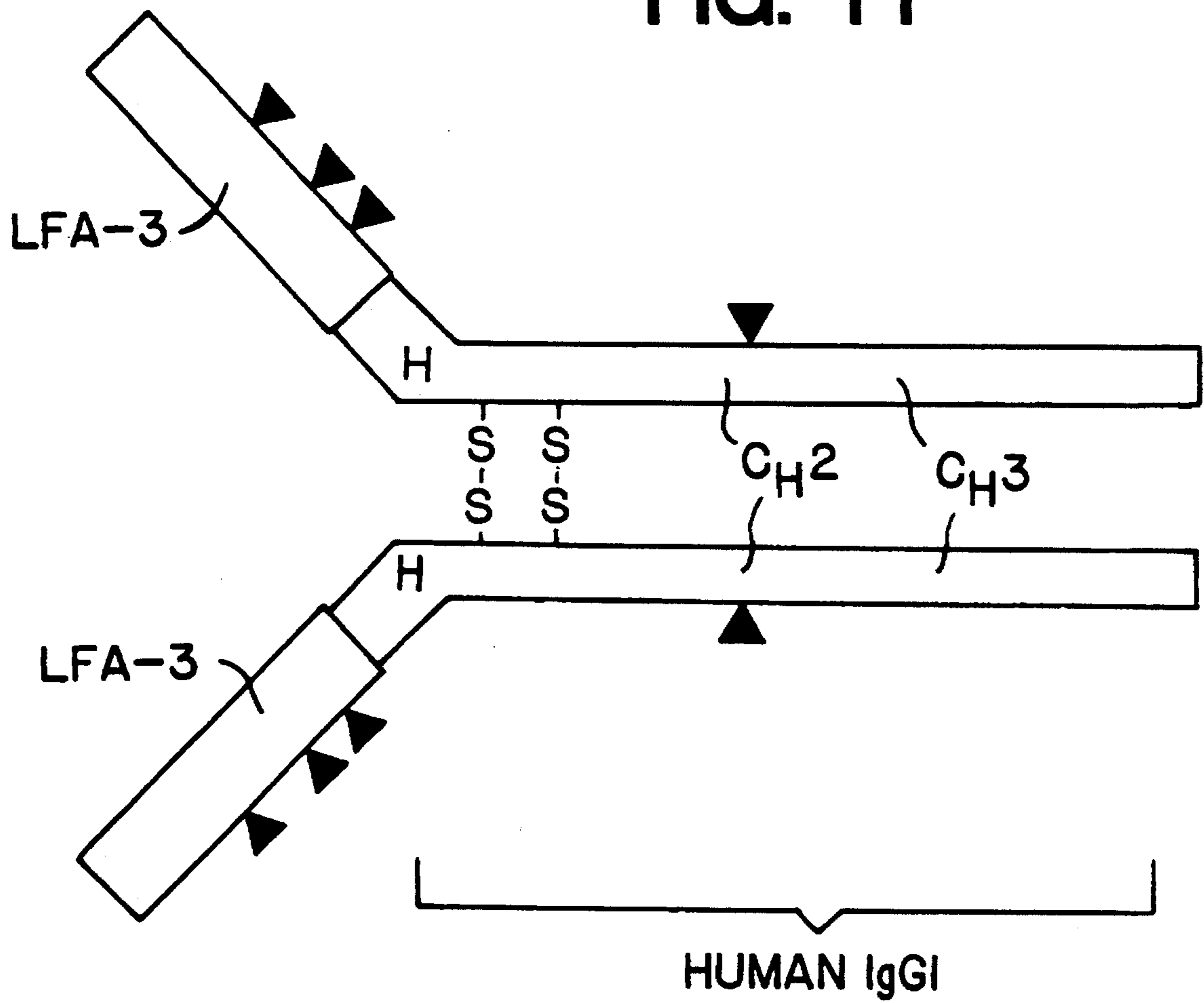


FIG. 12A 1

LFA-3 50
 ATGGTTGGAGCGACGGGGCCCTGGGGTCCCTCAGCGTGGT
 MetValAlaGlySerAspAlaGlyArgAlaLeuGlyValLeuSerValVa

51 100
 CTGCCCTGCTGCACTGCTTTGGTTTCATCAGCTGTTTCCCAACAATAT
 ICysLeuLeuHisCysPheGlyPheIleSerCysPheSerGlnGlnIleT

101 150
 ATGGTGTGTGTATGGGAATGTAACCTTCCATGTACCAAGCAATGTGCCT
 YrGlyValValTyrGlyAsnValThrPheHisValProSerAsnValPro

151 200
 TTAAAAGAGGTCCTATGGAAAACAAGGATAAAGTTCAGAACTGGA
 LeuLysGluValLeuTyrLysLysGlnLysAspLysValAlaGluLeuGI

201 250
 AAATTCTGAATTCAGAGCTTCTCATCTTTTAAAAATAGGGTTATTAG
 uAsnSerGluPheArgAlaPheSerSerPheLysAsnArgValTyrLeuA

251 300
 AACTGTGCAGGTAGCCTCACTATCTACAACCTAACATCATCAGATGAA
 spThrValSerGlySerLeuThrIleTyrAsnLeuThrSerSerAspGlu

301 350
 GATGAGTATGAAATGGAAATCGCCAAATAATTACTGATACCATGAAGTTCTT
 AspGluTyrGluMetGluSerProAsnIleThrAspThrMetLysPhePh
LFA-3 ← **Hinge** → **CH2**

351 400
 TCTTTATGTCGACAAAACCTCACACATGCCACCCTGCCAGCACCTGAAC
 eLeuTyrValAspLysThrHisThrCysProProCysProAlaProGluL

401 450
 TCCTGGGGACCGTCAGTCTTCTCTTCCCCAAACCCAAGGACACC
 euLeuGlyGlyProSerValPheLeuPheProProLysProLysAspThr

451 500
 CTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGACGTGAG
 LeuMetIleSerArgThrProGluValThrCysValValValAspValSe

501 550
 CCACGAAGACCCCTGAGGTC AAGTCAACTGGTACGTGGACGGCGTGGAGG
 rHisGluaspProGluValLysPheAsnTrpTyrValAspGlyValGluV

FIG. 12B

551	TGCATAATGCCAAGACAAAGCCGGGAGGAGCAGTACAACAGCACGTAC alHisAsnAlaLysThrLysProArgGluGlnTyrAsnSerThrTyr	600
601	CGGGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAA ArgValValSerValLeuThrValLeuHisGlnAspTrpLeuAsnGlyLy	650
651	GGAGTACAAGTGCAAGGTCTCCAACAAGCCCTCCAGCCCCATCGAGA sGluTyrLysCysLysValSerAsnLysAlaLeuProAlaProIleGluL ysThrIleSerLysAlaLysGlyGlnProArgGluProGlnValTyrThr	700
701	AAACCATCTCCAAGCCAAAGGGCAGCCCGAGAACACAGGTGTACACC ysThrIleSerLysAlaLysGlyGlnProArgGluProGlnValTyrThr	750
751	CTGCCCCCATCCCGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCCTG LeuProProSerArgaspGluLeuThrLysAsnGlnValSerLeuThrCy	800
801	CCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTGGAGTGGAGAGCA sLeuValLysGlyPheTyrProSerAspIleAlaValGluTrpGluSerA	850
851	ATGGGCAGCCGGAGAACAACACTACAAGACCACGCCCTCCCGTGTGACTCC snGlyGlnProGluAsnAsnTyrLysThrThrProProValLeuAspSer	900
901	GACGGCTCCTTCTCCTACAGCAAGCTCACCGTGGACAAGAGCAGGTG AspGlySerPhePheLeuTyrSerLysLeuThrValAspLysSerArgTr	950
951	GCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACA pGlnGlnGlyAsnValPheSerCysSerValMetHisGluAlaLeuHisA	1000
1001	ACCACACTACAGCAGAAGAGCCTCTCCCTGTCTCCGGGTAATGAGTGCCGG snHisTyrThrGlnLysSerLeuSerLeuSerProGlyLys	1050



FIG. 13



FIG. 14A

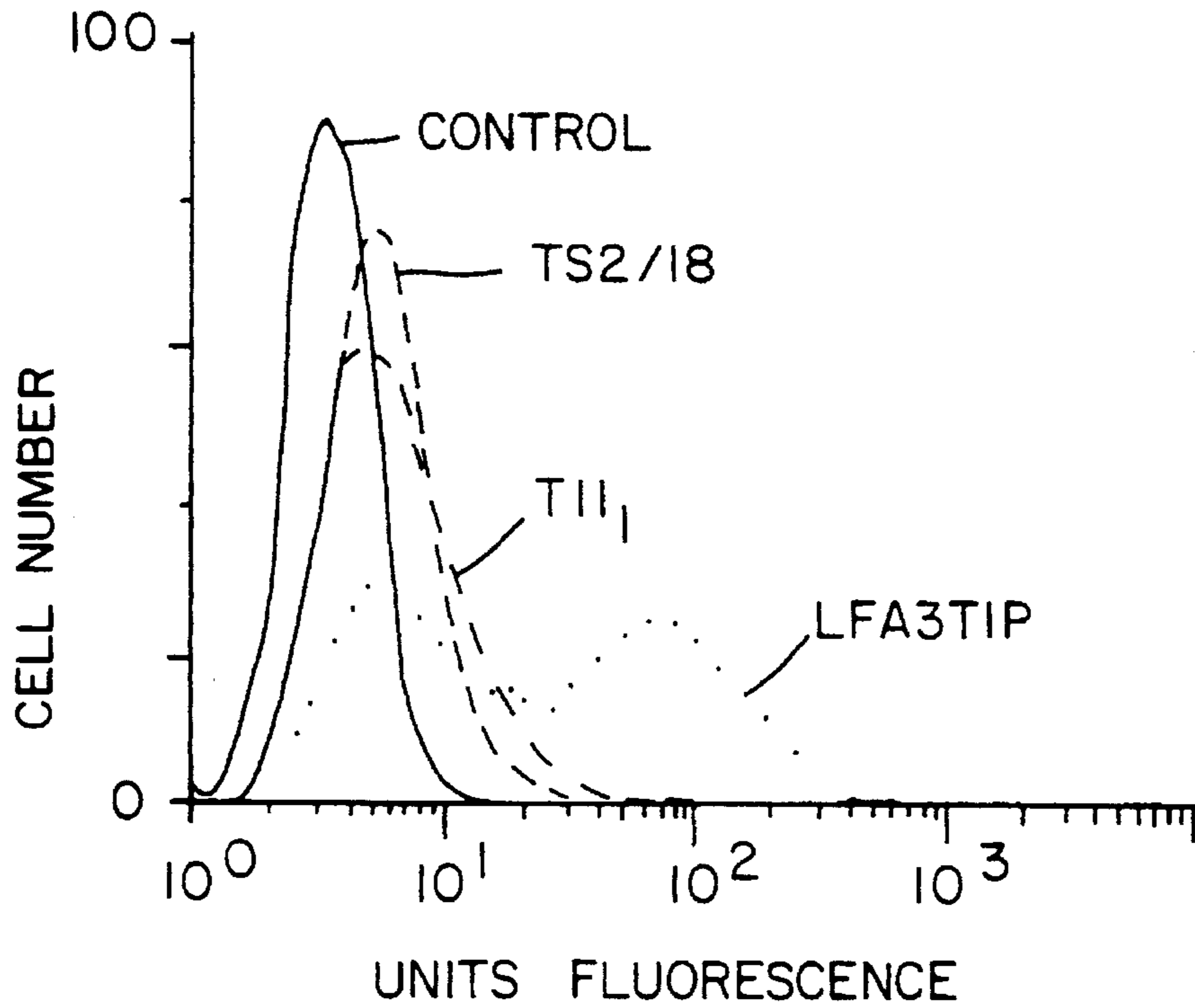


FIG. 14B

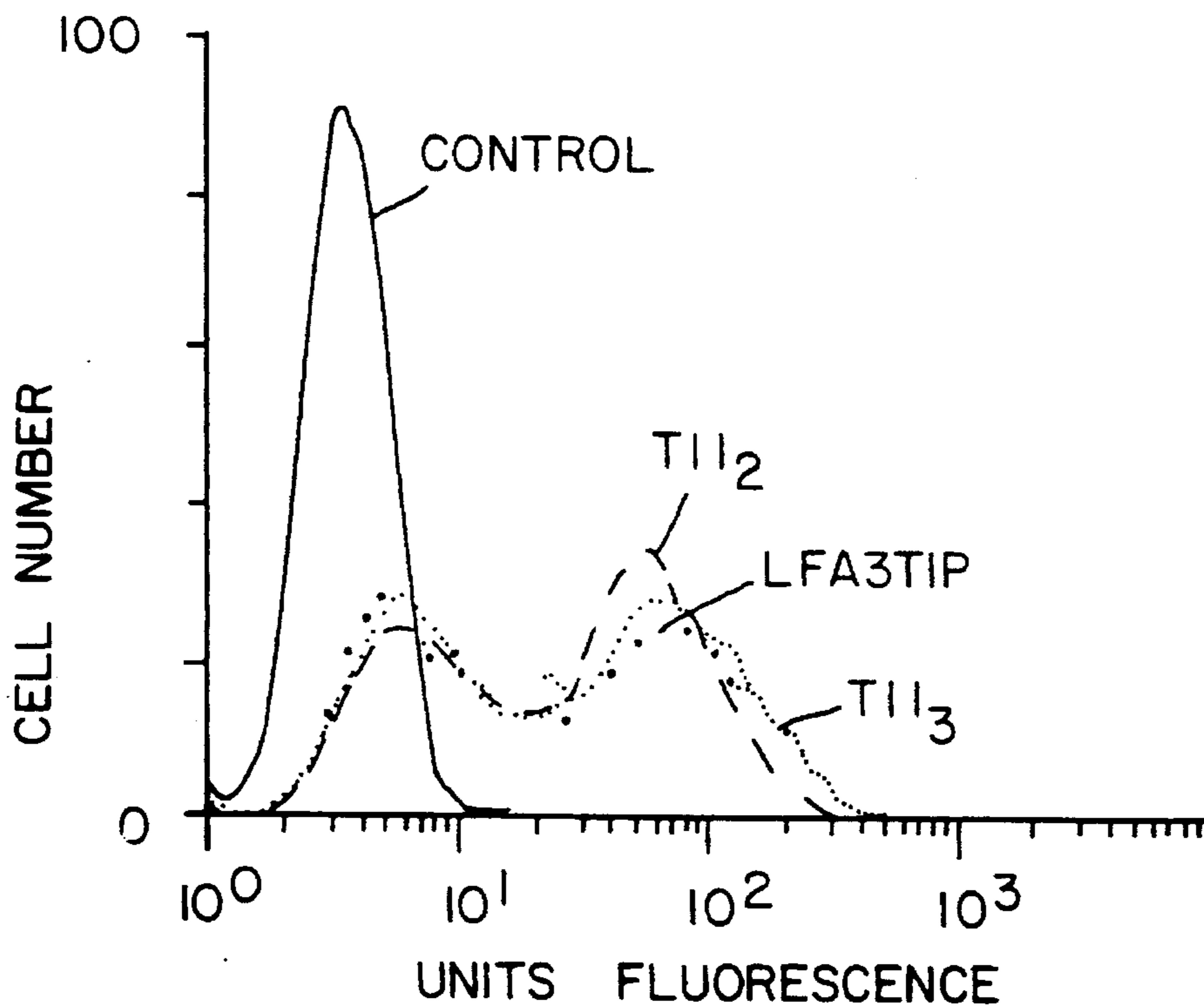


FIG. 15

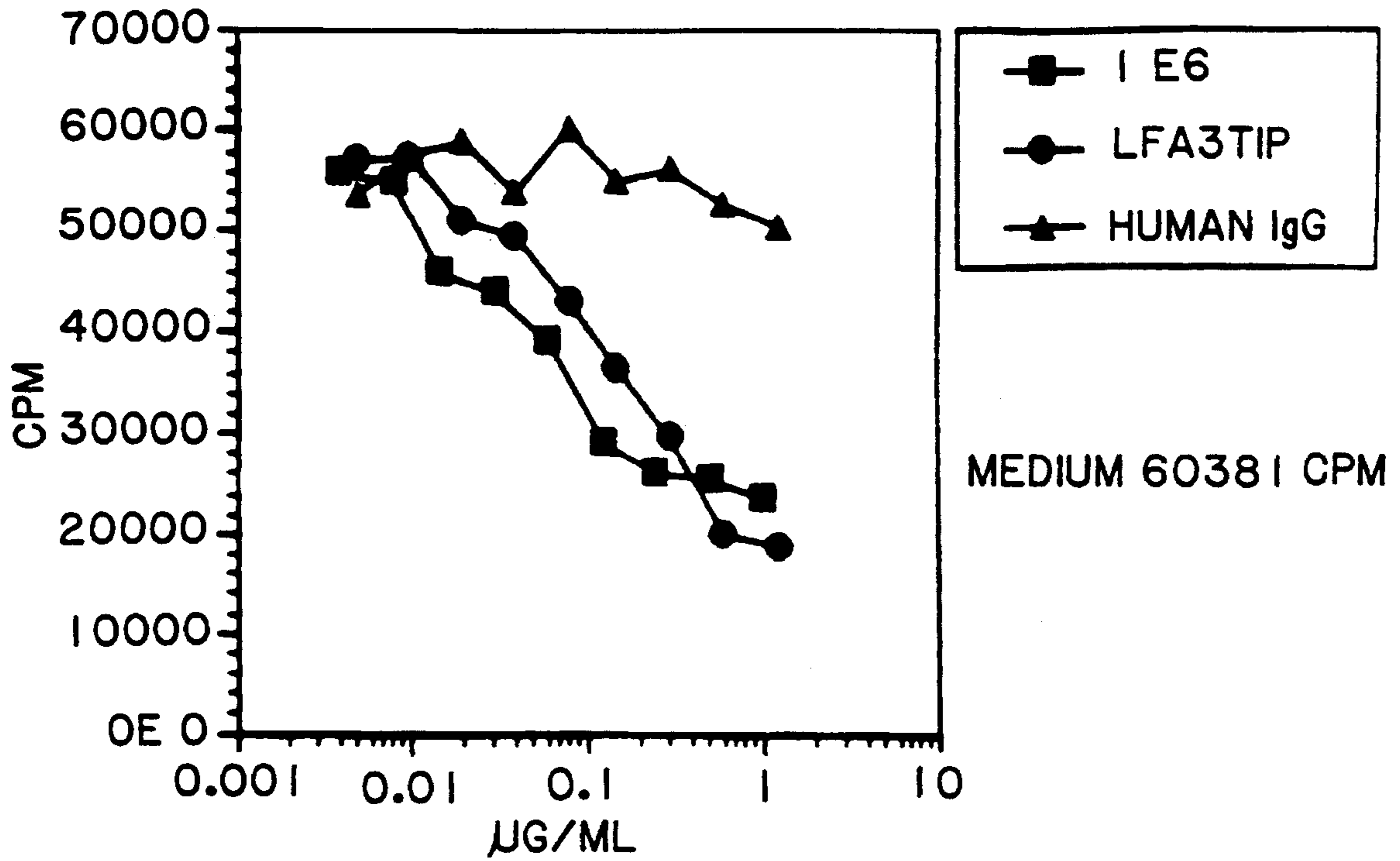


FIG. 16

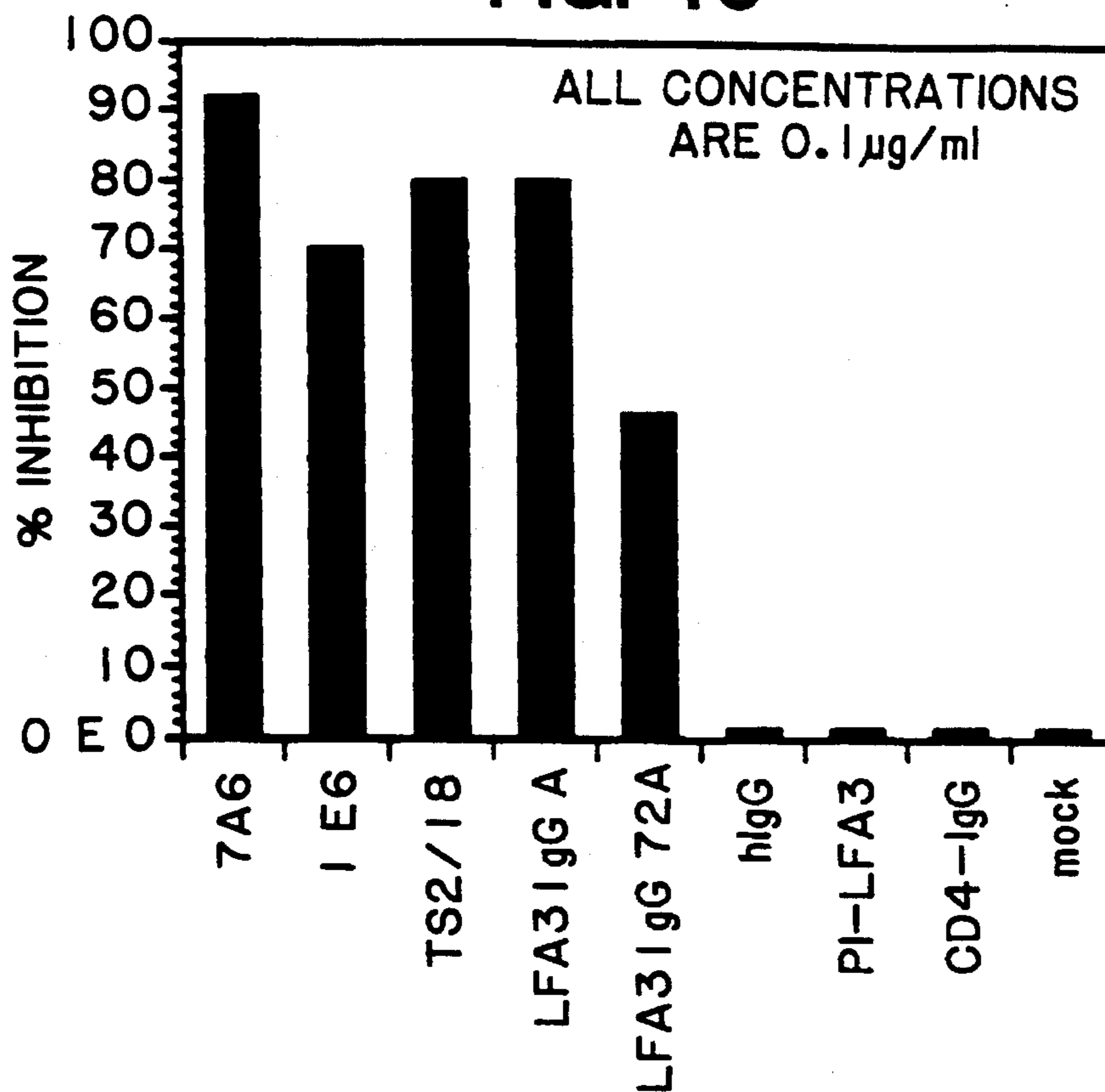
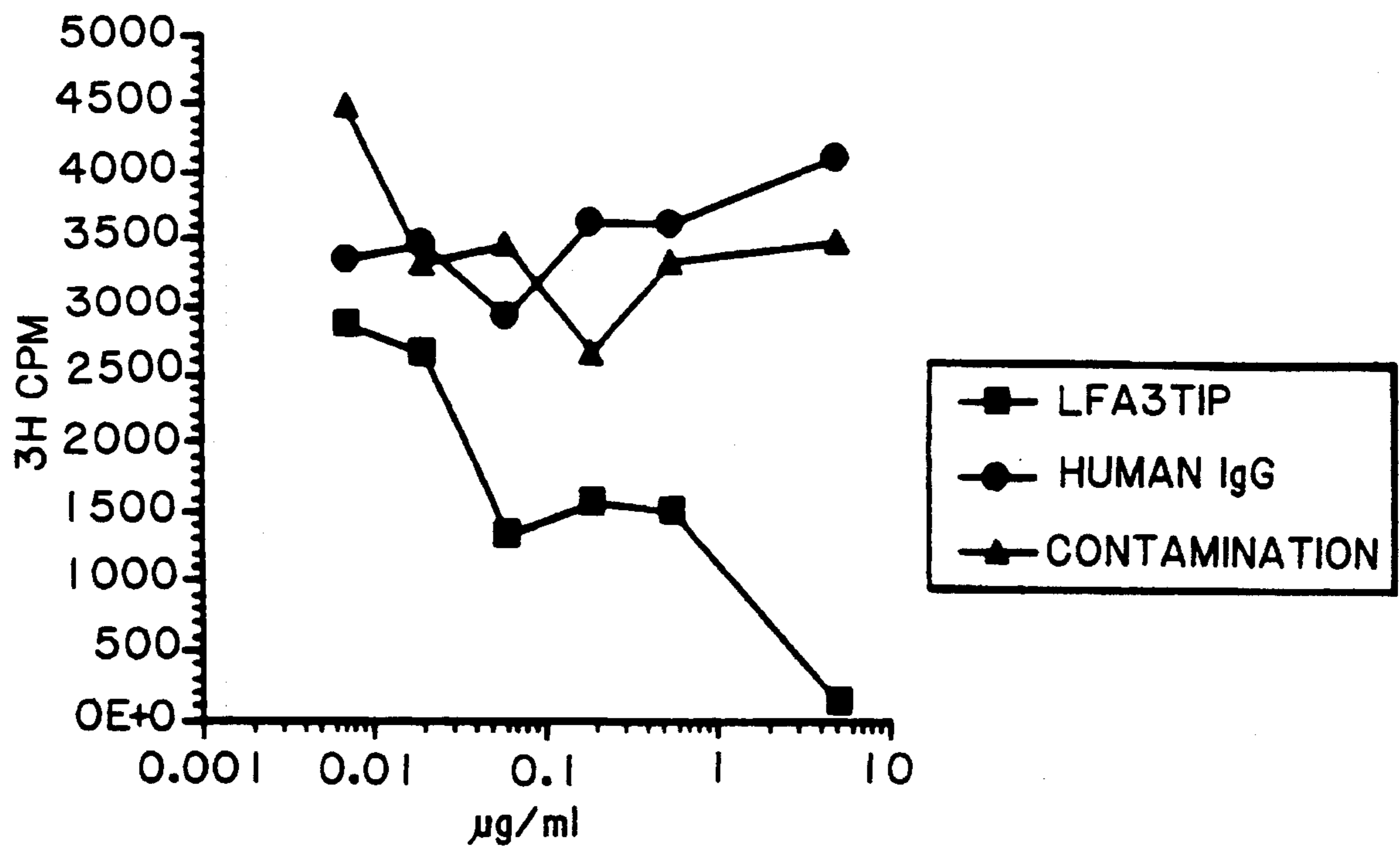


FIG. 17



MEDIUM 3337 cpm
T11 2+3 84936 cpm

FIG. 18

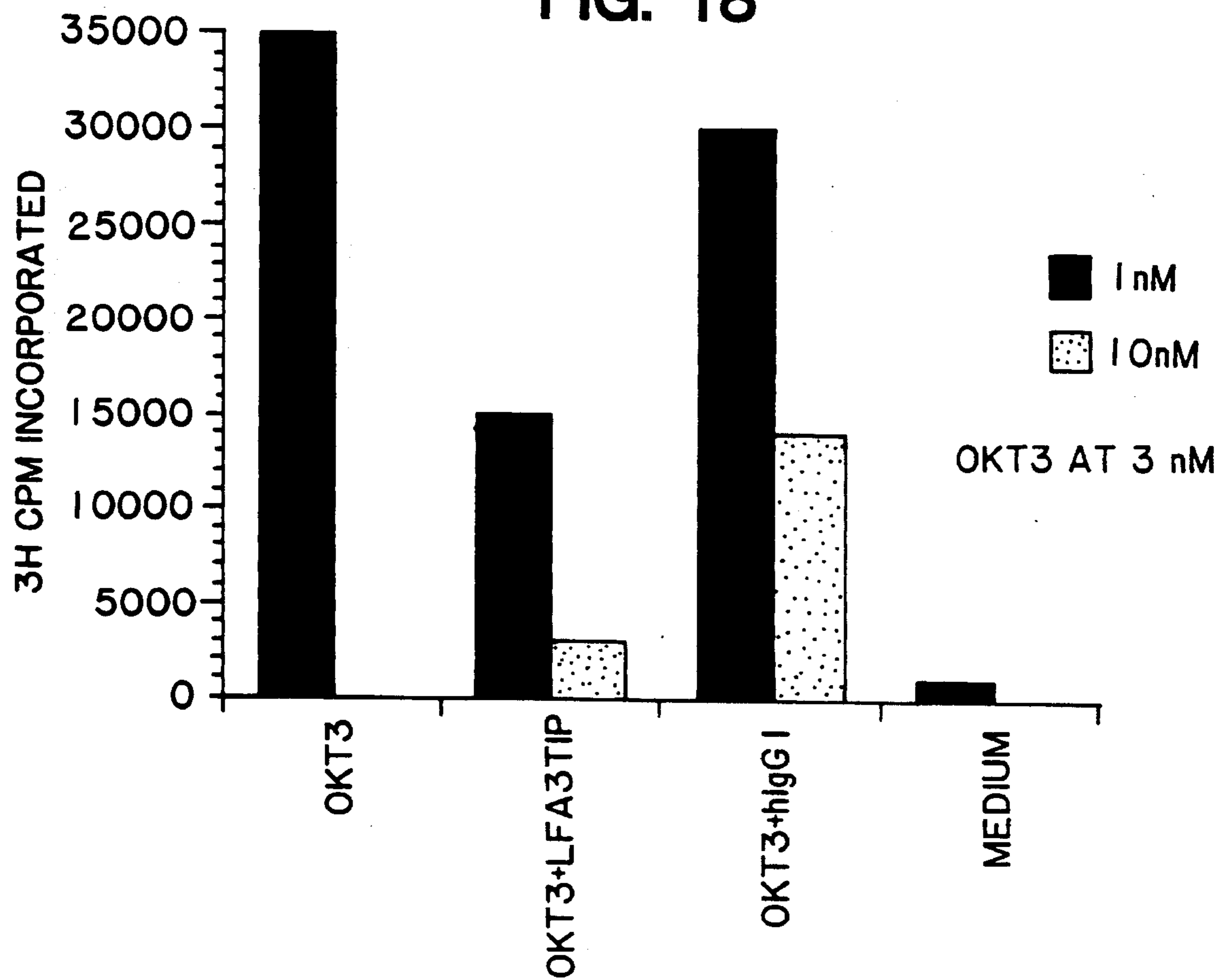
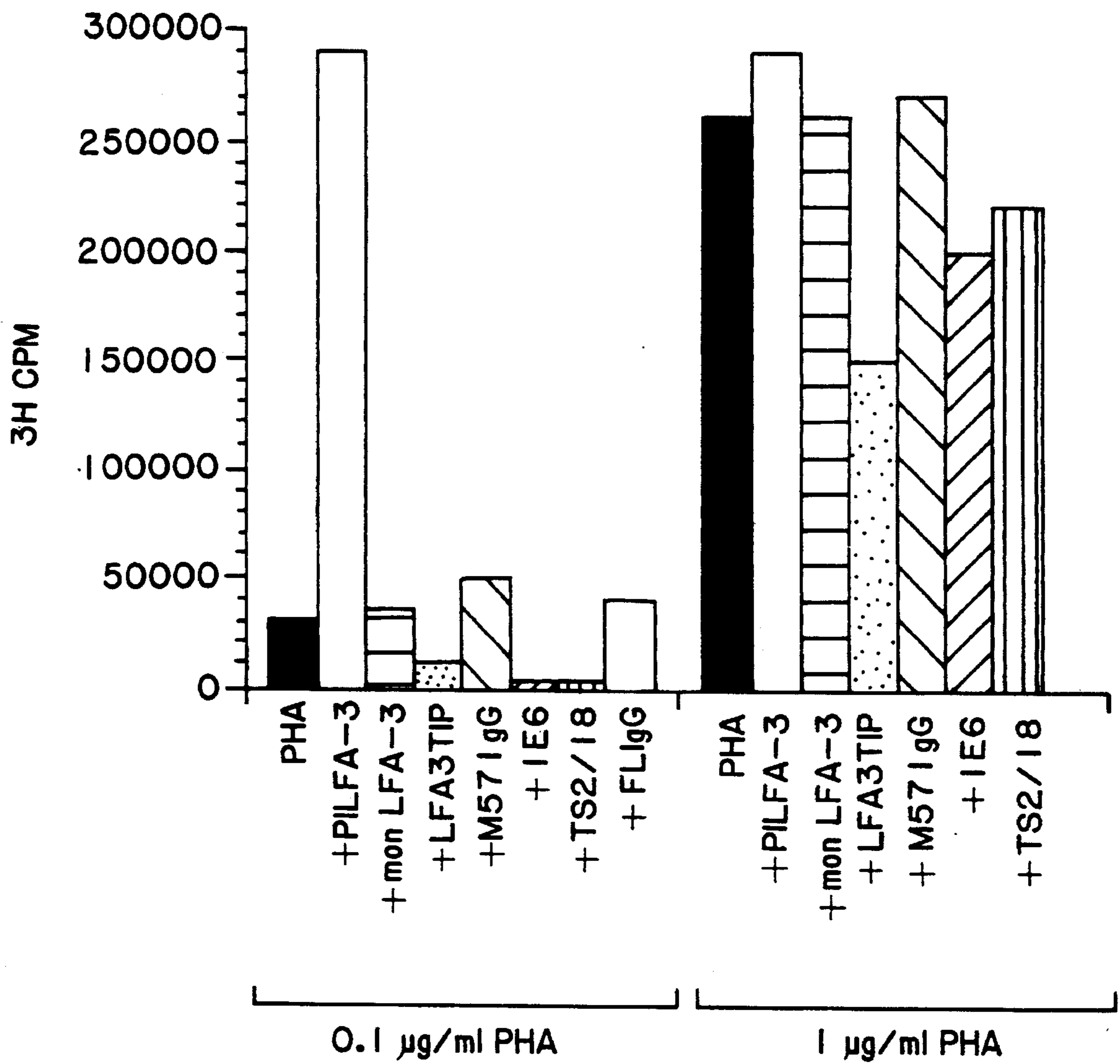


FIG. 19



CD2-BINDING DOMAIN OF LYMPHOCYTE FUNCTION ASSOCIATED ANTIGEN 3

This application is a continuation-in-part of application Ser. No. 07/770,967, filed Oct. 7, 1991, which is a continuation-in-part of prior application Ser. No. 07/667,971, filed Mar. 12, 1991, both now abandoned.

This invention relates to DNA sequences encoding and polypeptides which comprise the CD2-binding domain of Lymphocyte Function Associated Antigen 3 ("LFA-3") and to recombinant DNA molecules for expression of those polypeptides. In accordance with this invention, unicellular hosts transformed with the DNA sequences and recombinant DNA molecules containing these DNA sequences may be prepared and used to produce proteins and polypeptides comprising the CD2-binding domain of LFA-3. The peptides, polypeptides and proteins of this invention are useful in the study of interactions between CD2 and LFA-3, in diagnostic and therapeutic compositions, in antibody screening or purification methods and in other methods of this invention.

BACKGROUND OF THE INVENTION

T-lymphocytes play a major role in the immune response by interacting with target and antigen presenting cells. T-lymphocyte-mediated killing of target cells is a multi-step process involving adhesion of cytolytic T-lymphocytes to target cells. The initiation of the immune response to most antigens involves adhesion of helper T-lymphocytes to antigen-presenting cells.

These interactions of T-lymphocytes with target and antigen-presenting cells are highly specific and depend on the recognition of an antigen on the target or antigen-presenting cell by one of the many specific antigen receptors on the surface of T-lymphocytes.

The receptor-antigen interaction of T-lymphocytes and other cells is facilitated by various T-lymphocyte surface proteins, e.g., the antigen-receptor complex CD3 and accessory molecules CD4, LFA-1, CD8 and CD2. The interaction of T-lymphocytes and other cells is also dependent on accessory molecules, such as ICAM-1, MHC class I and II and LFA-3, that are expressed on the surface of target or antigen-presenting cells and thereby play a role in the action of T-lymphocytes. One general hypothesis is that accessory molecules on the T-lymphocytes and on the target or antigen-presenting cells interact with each other to mediate intercellular adhesion. Accordingly, these accessory molecules are believed to enhance the efficiency of lymphocyte/antigen-presenting cell and lymphocyte/target cell interactions and to be important in cell adhesion-based pathologies (such as leukocyte/endothelial cell interaction leading to pathologic inflammation) and lymphocyte recirculation. Accessory molecules are also involved in activation of lymphocytes.

One important example of cell-cell interaction mediated by accessory molecules is the specific interaction between CD2 (a T-lymphocyte accessory molecule) and LFA-3 (a target cell accessory molecule). CD2/LFA-3 binding appears to be essential for many important cell-cell reactions, including the initiation of the T-lymphocyte functional responses (Dustin et al., *J. Exp. Med.*, 165, pp. 677-92 (1987); Springer et al., *Ann. Rev. Immunol.*, 5, pp. 223-52 (1987)). The importance of the CD2/LFA-3 complex in cell-cell adhesion is indicated by the findings that purified LFA-3 binds to CD2 on the surface of T-lymphocytes (Dustin et al.,

J. Exp. Med., 165, pp. 677-92 (1987)), that CD2 purified from T-lymphocytes binds LFA-3 on cell surfaces and inhibits the binding of LFA-3-specific monoclonal antibodies ("MAbs") to LFA-3 (Selvaraj et al., *Nature*, 326, pp. 400-403 (1987)), and that rosetting of human erythrocytes, which express LFA-3, to cells expressing CD2 is blocked by anti-LFA-3 MAbs and anti-CD2 MAbs (see, e.g., Seed et al., *Proc. Natl. Acad. Sci. USA*, 84, pp. 3365-69 (1987)).

LFA-3, which is found on the surface of a wide variety of cells including monocytes, granulocytes, T-lymphocytes, erythrocytes, B-lymphoblastoid cell lines, thymic epithelial cells, and vascular endothelial cells, has become the subject of a considerable amount of study to further elucidate its role in various T-lymphocyte interactions. Two natural forms of LFA-3 have been identified. One form of LFA-3 ("transmembrane LFA-3") is anchored in the cell membrane by a transmembrane hydrophobic domain. cDNA encoding this form of LFA-3 has been cloned and sequenced (see, e.g., Wallner et al., *J. Exp. Med.*, 166, pp. 923-32 (1987)). Another form of LFA-3 is anchored to the cell membrane via a covalent linkage to phosphatidylinositol ("PI")-containing glycolipid. This latter form has been designated "PI-linked LFA-3", and cDNA encoding this form of LFA-3 has also been cloned and sequenced (Wallner et al., PCT patent application WO 90/02181).

Although the DNA sequence of the LFA-3 gene and the primary amino acid sequences of both forms of LFA-3 have been determined, the actual site of interaction between LFA-3 and its receptor, CD2, has not previously been identified. There is a need to identify the CD2-binding domain on LFA-3 in order to better understand the specific interaction between CD2 and LFA-3 and, thereby, to effect and modulate the cellular and immunological processes that are dependent on the formation of the CD2/LFA-3 complex. Such information would also be useful in a variety of other applications including diagnostic and therapeutic compositions, protein purification, antibody identification and purification, and comparative and structural studies of LFA-3 and other proteins.

SUMMARY OF THE INVENTION

This invention addresses the aforementioned needs by identifying the CD2-binding domain of LFA-3 and providing nucleotide sequences defining the CD2-binding region of LFA-3 and CD2 binding polypeptides encoded by those sequences. The present invention provides polypeptides having the amino acid sequence: X₁-X₂-(SEQ ID NO: 1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 32 amino acids of the sequence (SEQ ID NO: 33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;

and analogs and derivatives thereof, said polypeptides being capable of binding to CD2.

In another embodiment, the present invention provides polypeptides having the amino acid sequence: X₁-X₂-(SEQ ID NO: 1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 10 amino acids of the sequence (SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser;

and analogs and derivatives thereof, said polypeptides being capable of binding to CD2.

The invention also provides DNA sequences encoding the above polypeptides, recombinant molecules containing those DNA sequences, hosts transfected with such DNA sequences and molecules and methods of making the polypeptides recombinantly, synthetically or semi-synthetically.

The polypeptides of this invention are useful in diagnosing diseases characterized by the presence or absence of CD2 on the surface of particular cells and to treat pathologies dependent on the formation of the LFA-3/CD2 complex.

The polypeptides of this invention and the DNA sequences encoding them may also be used to prepare recombinant or synthetic fusion proteins, which comprise a functional CD2-binding domain or polypeptide of LFA-3, as defined above, and another domain of a protein or polypeptide other than LFA-3. The CD2-binding domain portion of the fusion proteins allows the other polypeptides to be targeted specifically to CD2-expressing cells (CD2⁺ cells). DNA sequences encoding these fusion proteins are also part of this invention.

One example of such fusion proteins of this invention is novel fusion proteins containing a portion of LFA-3 containing a functional CD2-binding domain, as defined above, fused to at least a portion of the Fc region of an immunoglobulin (Ig). Unexpectedly, the LFA-3-Ig fusion proteins of this invention inhibit T-lymphocyte activation and proliferation of peripheral blood lymphocytes.

It is a further aspect of this invention that the polypeptides and fusion proteins of this invention may also be used to label CD2 molecules, for example, soluble CD2, in solution or on the surface of T-lymphocytes or other CD2-expressing cells. The polypeptides and fusion proteins of this invention may also be used to label any protein, polypeptide or peptide comprising the domain of CD2 which binds LFA-3, for example, in a fusion protein generated using recombinant DNA techniques.

The polypeptides and fusion proteins of this invention may also be used in affinity chromatography to purify CD2 or any other polypeptide or protein conjugate containing the domain of CD2 which binds LFA-3.

This invention also provides altered (mutant) forms of the LFA-3 protein which lack the CD2-binding domain and the DNA sequences encoding such "deletion mutant" forms of LFA-3. Mutant proteins, which lack the CD2-binding domain of LFA-3, as defined above, may be used in vivo or in vitro to generate or to bind antibodies or other molecules

that recognize epitopes or sites on LFA-3 other than the CD2-binding domain.

In addition to the monomeric form of the CD2-binding polypeptides and fusion proteins of this invention, multimeric forms are also enabled by this invention. Such forms may have enhanced affinity for CD2, enhanced immunogenicity and/or enhanced ability to initiate T-lymphocyte functional responses, e.g., stimulation of T-cell activation, through more effective or multiplied formation of CD2/LFA-3 complexes. Also, such multimeric forms may be more effective in competitive binding of CD2, making them more useful as immunosuppressants and more sensitive as diagnostics or reagents.

In addition, this invention contemplates antibodies recognizing the polypeptides and fusion proteins of this invention. Polyclonal and monoclonal antibodies to the polypeptides and fusion proteins of this invention may be obtained by immunizing an animal with polypeptides or fusion proteins of this invention.

Low molecular weight (generally less than 1000 daltons) inhibitors of the formation of the CD2/LFA-3 complex are also provided by this invention. Such "small molecule" inhibitors may be produced in vitro by synthetic methods and may comprise part of the amino acid sequences of the polypeptides of this invention or may be entirely non-peptidyl organic molecules having a structural conformation that is able to mimic the binding specificity of the polypeptides and fusion proteins of this invention to CD2.

This invention also provides methods, compositions and kits for diagnostic and therapeutic uses in which the presence or absence of the CD2-binding domain of LFA-3 is desirable.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1, 1A-2 and 1B taken together depict amino acid (SEQ ID NO:10) and nucleotide (SEQ ID NO:9) sequences of transmembrane LFA-3 indicating (with underlining) deletions made in the extracellular region of LFA-3. Regions designated M53, M54, M55, M56, M57, M58, M59, M60, M61, M62, M63, M64, M65, M66, M90, M91 and M92, respectively, were looped out to create recombinant genes encoding 17 separate deletion mutants. Expression of the deletion mutants in mammalian cells provided altered LFA-3 surface proteins missing a segment of amino acids present in the native LFA-3 protein. For example, expression of the deletion mutant designated M57 (that is, the LFA-3 DNA of FIGS. 1A-1 and 1A-2 having the M57 region deleted) in CHO cells resulted in a mutant LFA-3 that did not bind to CD2. The amino terminal methionine (Met) is designated -28 to indicate that it is the amino terminal residue of the 28-amino acid signal peptide sequence of preLFA-3. The amino terminal residue of mature LFA-3 is a phenylalanine (Phe) and is designated +1.

FIGS. 2A, 2B, 2C, 2D, 2E, 2F, 2G, 2H, 2I, 2J, 2K, 2L, 2M and 2N taken together depict results of immunofluorescence flow cytometry by FACS analysis of transfected CHO cells expressing mutant forms of LFA-3 encoded by deletion mutants M57 (FIGS. 2A and 2B), M65 (FIGS. 2C and 2D), M63 (FIGS. 2E and 2F), M54 (FIGS. 2G and 2H), M55 (FIGS. 2I and 2J), M56 (FIGS. 2K and 2L), and M58 (FIGS. 2M and 2N). Transfected cells were reacted with anti-LFA-3 polyclonal antiserum 202 (dashed lines, FIGS. 2A, 2C, 2E, 2G, 2I, 2K, 2M), anti-LFA-3 MAb TS2/9 (dashed lines, FIGS. 2B, 2D, 2F, 2H, 2J, 2L, 2N), or anti-LFA-3 MAb 7A6 (dotted lines, FIGS. 2B, 2D, 2F, 2H, 2J, 2L, 2N). In each

analysis, the control peak (solid line) represents the cell population with background levels of fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse or goat anti-rabbit IgG binding.

FIGS. 3A, 3B and 3C taken together depict the results of a Jurkat cell binding experiment. The photomicrographs show Jurkat cells expressing CD2 mixed with normal CHO cells (negative control) (FIG. 3A), M57/CHO cells (FIG. 3B) and P24/CHO cells expressing PI-linked LFA-3 (positive control) (FIG. 3C).

FIG. 4 depicts a Northern gel analysis of LFA-3 gene transcripts synthesized in M57/CHO cells. Filters containing mRNA samples from CHO negative control cells (lane 1), M57/CHO cells (lane 2) and M16.3/CHO positive control cells (lane 3) were probed with a NotI restriction fragment of plasmid pLFA3M54, containing nucleotides 1-153 and 184-1040 of the LFA-3 cDNA sequence (see FIGS. 1A-1, 1A-2 and 1B), labeled with 32 P by nick-translation.

FIG. 5 shows an autoradiogram depicting the differential immunoprecipitation of proteins from 125 I surface-labeled M57/CHO cells (lanes 1-4), normal CHO cells (negative control) (lanes 5-7), and P24/CHO cells (positive control) (lanes 8-11). Lysates of surface-labeled cells were reacted with anti-LFA-3 MAb TS2/9 (lanes 2, 5, 9), anti-LFA-3 MAb 7A6 (lanes 3, 6, 10), or anti-LFA-3 polyclonal antiserum 202 (lanes 4, 7, 11). The antibody-precipitated proteins were electrophoresed on SDS-polyacrylamide gels prior to autoradiography.

FIGS. 6A and 6B taken together depict amino acid (SEQ ID NO:10) and nucleotide (SEQ ID NO:9) sequences of transmembrane LFA-3 indicating (with underlining) deletions in the extracellular region of LFA-3. Regions designated M100, M101, and M102, respectively, were looped out to create recombinant genes encoding 3 separate deletion mutants.

FIGS. 7A, 7B, 7C and 7D taken together show results of Jurkat cell-binding experiments in which Jurkat cells expressing CD2 were mixed with (a) normal CHO cells, (b) M100/CHO cells, (c) M101/CHO cells, or (d) M102/CHO cells.

FIGS. 8A, 8B and 8C taken together show Jurkat cell binding by LFOS/Affigel-10 beads (see Example 8, *infra*). LF08/Affigel-10 beads were mixed with Jurkat cells and the cell-bead binding observed under a microscope (FIGS. 8A and 8B). For comparison (negative control), a non-LFA-3 peptide from hepatitis B (i.e., "MXC 01", (SEQ ID NO:4) Thr Lys Pro Asp Leu Val Asp Lys Gly Thr Glu Asp Lys Val Val Asp Val Val Arg Asn) was fixed to Affigel-10 beads, the beads mixed with Jurkat cells, and cell-bead binding recorded in FIG. 8C.

FIGS. 9A and 9B depict the amino acid (SEQ ID NO:12) and nucleotide (SEQ ID NO:11) sequences of PI-linked LFA-3 indicating (with underlining) the internal deletion made in the nucleotide sequence and corresponding mutant PI-linked LFA-3 protein. The region of the DNA designated PIM3 was looped out to create a recombinant gene encoding a mutant protein containing the N-terminal 89 amino acids of PI-linked LFA-3 but lacking the subsequent 71 amino acids. Expression of the deletion mutant PIM3 (that is, the PI-linked LFA-3 DNA of FIGS. 9A and 9B having the PIM3 region deleted) in CHO cells resulted in a mutant form of PI-linked LFA-3 that retained a CD2-binding domain.

FIGS. 10A and 10B taken together depict results of immunofluorescence flow cytometry by FACS analysis of transfected CHO cells expressing a mutant form of PI-linked LFA-3 encoded by deletion mutation PIM3. Transfected

cells (PIM3.25.2 cells) expressed a surface protein that was recognized by anti-LFA-3 MAb TS2/9 (FIG. 10A, dashed line) and that was susceptible to release from the cell surface by PI-PLC treatment (FIG. 10A, dotted line). FIG. 10B depicts the results of a similar analysis using transfected CHO cells amplified for expression of the mutant PI-linked protein (PIM2.25.2.100.12 cells). These cells produced a protein on their cell surface recognized by MAb 7A6 (FIG. 10B, dashed line) and that was susceptible to release from the cell surface by PI-PLC treatment (FIG. 10B, dotted line). In each analysis, the control peak (solid line) represents the cell population with background levels of FITC-conjugated goat anti-mouse IgG binding.

FIG. 11 is a diagram of a dimer of LFA3TIP, depicting the various domains of this LFA-3-Ig fusion protein. As depicted in this figure, "LFA-3" refers to the amino terminal 92 amino acids of mature LFA-3. "H" refers to the ten amino acids of the IgG1 hinge region containing two cysteines to form two intermolecular disulfide bonds. Each disulfide bond is depicted as horizontal double SS. "C_H2" and "C_H3" refer to the two constant domains present in the Fc region, below the hinge region, in human IgG1 molecules.

FIGS. 12A and 12B depict the amino acid (SEQ ID NO:43) and nucleotide (SEQ ID NO:42) sequences of preLFA3TIP (i.e., LFA-3 (amino acids +1-+92) and the 28-amino acid signal sequence) and the various domains of the fusion protein. The nucleotide sequence of FIGS. 12A and 12B is also the same as the DNA sequence insert in the expression plasmid pSAB152.

FIG. 13 is a photograph of a Western blot analysis of SDS-PAGE gels under nonreducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions. Lanes 1 and 3 are high molecular weight markers (BRL, Gaithersburg, Md.). Lanes 2 and 4 contain LFA3TIP purified as described in Example 13.

FIGS. 14A and 14B taken together depict results of immunofluorescence flow cytometry by FACS analysis of Jurkat cells incubated with LFA3TIP plus R-Phycoerythrin conjugated anti-human IgG F(ab')₂ (dotted lines) in the presence of anti-CD2 MAb TS2/18 plus FITC conjugated goat anti-mouse IgG(H+L) F(ab')₂ (small dots in FIG. 14A) or anti-CD2 ascites fluid T11₁ (dashes in FIG. 14A), T11₂ (dashes in FIG. 14B) or T11₃ (small dots in FIG. 14B), plus FITC conjugated goat anti-mouse IgG(H+L) F(ab')₂.

FIG. 15 depicts the results of a human allogeneic mixed lymphocyte reaction (MLR) assay for T-cell activation as measured by 3 H-thymidine incorporation into T-cells in the presence of anti-LFA-3 MAb 1E6 (closed squares), LFA3TIP (closed circles) and nonspecific human IgG1 (closed triangles).

FIG. 16 depicts the results of MLR assays for inhibition of T-cell activation by selected proteins. "7A6" and "1E6" are anti-LFA-3 monoclonal antibodies (MAbs), specific for the CD2-binding domain of LFA-3. "TS2/18" is an anti-CD2 MAb. "LFA3IgGA" and "LFA3IgG72A" are preparations of LFA3TIP differing in purity, i.e., 75% and 50%, respectively. hIgG is nonspecific human IgG. "PI-LFA3" is multimeric PI-linked LFA-3. "CD4-IgG" is a fusion protein consisting of a portion of the CD4 protein fused to a portion of the Fc region of IgG. "Mock" refers to a "mock preparation" purified from COS7 cells transfected with an expression vector pSAB132 lacking the DNA sequence encoding LFA3TIP.

FIG. 17 depicts results of a PBL proliferation assay as measured by incorporation of 3 H-thymidine. PBL proliferation was measured in the presence of LFA3TIP (closed

squares), human IgG (closed circles) and a "mock preparation" containing the contaminant ("contamination") which co-purifies with the LFA3TIP used in this assay (closed triangles).

FIG. 18 depicts results of an OKT3 (anti-CD3 MAb) dependent PBL proliferation assay as measured by incorporation of ³H-thymidine. PBL proliferation was measured in the presence of 3 nM OKT3 (OKT3), 3 nM OKT3 plus 1 nM or 10 nM LFA3TIP (OKT3 + LFA3TIP), 3 nM OKT3 plus 1 nM or 10 nM human IgG1 (OKT3 + hIgG1), or medium with no OKT3 present (medium).

FIG. 19 depicts results of a phytohemagglutinin (PHA) dependent PBL proliferation assay as measured at a suboptimal stimulatory concentration (0.1 µg/ml) and at an optimal stimulatory concentration (1 µg/ml) of PHA. PBL proliferation was measured as indicated in the presence of PHA alone (PHA) and in the presence of PEA plus: PI-linked LFA-3 (+PILFA-3); monomeric soluble LFA-3 (+mon LFA-3); LFA3TIP (+LFA3TIP); an LFA-3-IgG fusion protein which lacks the 10 amino acid M57 region of LFA-3 involved in CD2 binding (+M57IgG); anti-LFA-3 1E6 MAb (+1E6); anti-CD2 TS2/18 MAb (+TS2/18); and a full-length LFA-3-Ig fusion protein (+FLIgG). Each of the added molecules was present at 5 µg/ml.

DETAILED DESCRIPTION OF THE INVENTION

The polypeptides, compositions, and methods of the present invention are characterized by polypeptides having the amino acid sequence: X₁-X₂-(SEQ ID NO: 1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;

and analogs and derivatives thereof, said polypeptides being capable of binding to CD2.

In another embodiment, the polypeptides of this invention have the amino acid sequence: X₁-X₂ (SEQ ID NO: 1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 10 amino acids of the sequence (SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser;

and analogs and derivatives thereof, said polypeptides being capable of binding to CD2.

Preferably, the polypeptides of this invention have the amino acid sequence of amino acids 29-120 of SEQ ID NO:10, amino acids 29-108 of SEQ ID NO:10, amino acids 48-108 of SEQ ID NO:10, and SEQ ID NO:7.

Throughout this specification and in the claims, the abbreviations employed for amino acids and their residues are used in conformity with the generally accepted rules of nomenclature and relate to α-amino acids and their residues of the L-series.

A derivatized amino acid is a natural or nonnatural amino acid in which the normally occurring side chain or end group is modified by chemical reaction. Such modifications include, for example, gamma-carboxylation, β-hydroxylation, sulfation, sulfonation, phosphorylation, amidization, esterification, t-butoxy carbonylation, N-acetylation, carbobenzoxylation, tosylation, benzylation, and other modifications known in the art.

A derivatized polypeptide is a polypeptide containing one or more derivatized amino acids.

Analogous of the polypeptides of this invention may be characterized, for example, by amino acid substitutions, additions or deletions, or utilization of D-amino acids. The preferred substitutions in the polypeptides of this invention are those that are recognized in the art to be conservative amino acid substitutions. For example, amino acids belonging to one of the following groups represent conservative changes: ala, pro, gly, glu, asp, gln, asn, ser, thr; cys, ser, tyr, thr; val, ile, leu, met, ala, phe; lys, arg, his; and phe, tyr, trp, his. See, e.g., Grantham, *Science*, 185, pp. 862-64 (1974); Dayhoff, In *Atlas of Protein Sequence and Structure*, 5, 1978; Argos, *EMBO J.*, 8, , pp. 779-785 (1989).

It should be understood that all analogues and derivatives of this invention are characterized by biological activities that are similar to those of the CD2-binding polypeptides described herein. Accordingly, these analogs and derivatives may be employed in compositions, combinations and methods for diagnosis, therapy and prophylaxis in the same manner as the polypeptides of this invention.

The production of the polypeptides of this invention may be achieved by a variety of methods known in the art. For example, the polypeptides may be derived from the intact transmembrane or PI-linked LFA-3 molecules by proteolysis using specific endopeptidases in combination with exopeptidases, Edman degradation, or both. The intact LFA-3 molecules, in turn, may be purified from natural sources using conventional methods. Alternatively, full-length LFA-3 or truncated forms of LFA-3 may be produced by known recombinant DNA techniques using cDNAs. (See, U.S. Pat. No. 4,956,281 to Wallner et al.)

Preferably, the polypeptides of the present invention are produced directly, thus eliminating the need for a larger LFA-3 as a starting material. This may be achieved by conventional chemical peptide synthesis techniques or by well-known recombinant DNA techniques, wherein only those DNA sequences which encode the desired polypeptides are expressed in transformed hosts.

A gene which encodes the desired LFA-3 polypeptide of this invention may be designed based on the amino acid sequence of the desired polypeptide. Standard methods may be then applied to synthesize the gene. For example, the amino acid sequence may be used to construct a back-translated gene. A DNA oligomer containing a nucleotide sequence capable of coding for an LFA-3 polypeptide of this invention may be synthesized in a single step. Alternatively,

several smaller oligonucleotides coding for portions of the desired polypeptide may be synthesized and then ligated together. Preferably, the DNA sequence encoding an LFA-3 polypeptide of this invention will be synthesized as several separate oligonucleotides which are subsequently linked together. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled, preferred genes will be characterized by sequences that are recognized by restriction endonucleases (including unique restriction sites for direct assembly into a cloning or an expression vector), preferred codons taking into consideration the host expression system to be used, and a sequence which, when transcribed, produces a stable, efficiently translated RNA. Proper assembly may be confirmed by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a suitable host.

One embodiment of a DNA sequence according to this invention comprises the nucleic acid sequence encoding amino acids 1 through 70 of mature LFA-3, i.e., nucleotides 94 through 303 in FIGS. 1A and 1B. Another preferred embodiment comprises the nucleic acid sequence (SEQ ID NO:6): AATAGGGTTT ATTTAGACAC TGTGTCAGGT, which codes for amino acids 51-60 of mature LFA-3. However, the preferred DNA sequences of this invention encode polypeptides having the amino acid sequences of amino acids 29-120 of SEQ ID NO:10, amino acids 29-108 of SEQ ID NO:10, amino acids 48-108 of SEQ ID NO:10, and SEQ ID NO:7. The most preferred DNA sequence of this invention encodes a polypeptide having the amino acid sequence 1-120 of SEQ ID NO:10. This sequence when employed in animal cells allows production, secretion and maturation of a polypeptide having the amino acid sequence of amino acids 29-120 of SEQ ID NO:10.

It will be appreciated by those of skill in the art that, due to the degeneracy of the genetic code, DNA molecules comprising many other nucleotide sequences will also be capable of encoding the polypeptides of this invention. These degenerate sequences are encompassed by the present invention.

The present invention also relates to recombinant DNA molecules comprising the aforementioned DNA sequences. The recombinant DNA molecules of this invention are capable of directing expression of the LFA-3 polypeptides of this invention in hosts transformed therewith. A DNA sequence encoding an LFA-3 polypeptide of this invention must be operatively linked to an expression control sequence for such expression. The term "operatively linked" as used herein refers to positioning in a vector such that transcription and translation of the coding sequence is directed by the control sequence.

To construct a recombinant DNA molecule capable of directing expression of the LFA-3 polypeptides of this invention, the DNA sequences encoding these polypeptides may be inserted into and expressed using a wide variety of vectors. Furthermore, within each specific expression vector, various sites may be selected for insertion of these DNA sequences. These sites are usually designated by the restriction endonuclease which cuts them. They are well recognized by those of skill in the art. It will be appreciated, however, that an expression vector useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vector may be joined to the fragment by alternative means.

The expression vector, and in particular the site chosen for insertion of a selected DNA fragment and operative linking

to an expression control sequence, is determined by a variety of factors. These factors include, e.g., the number of sites susceptible to a particular restriction enzyme, the size of the polypeptide to be expressed, susceptibility of the desired polypeptide to proteolytic degradation by host cell enzymes, contamination or binding of the polypeptide to be expressed by host cell proteins difficult to remove during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those skilled in the art. The choice of a vector and an insertion site for a DNA sequence is determined by a balance of these factors and not all selections will be equally effective for a given case.

Useful expression vectors for eukaryotic hosts include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus, and vectors useful specifically in insect cells, such as pVL 941. Useful bacterial expression vectors include known bacterial plasmids, e.g., plasmids from *E. coli* including colE1, pCR1, pBR322, pMB9 and their derivatives; wider host range plasmids, such as RP4; the numerous derivatives of phage lambda, e.g., NM 989 and the lambda gt series; other DNA phages, e.g., M13 and other filamentous single-stranded DNA phages; and commercially available high expression vectors, e.g., the pGEM series and the lambda Zap vectors. Useful mammalian cell expression vectors include, for example, pNUT. Vectors useful in yeasts include, for example, the 2 μ plasmid and derivatives thereof.

Such expression vectors are also characterized by at least one expression control sequence that may be operatively linked to the DNA sequences of this invention inserted in the vector in order to control and to regulate the expression of that cloned DNA sequence. Examples of useful expression control sequences include the malE system, the OmpA system, the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the yeast acid phosphatase, e.g., Pho5, the promoters of the yeast mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, eukaryotic cell promoters, such as the metallothionein promoter and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

The recombinant DNA molecules of the present invention may also comprise other DNA coding sequences fused to and in frame with the DNA sequences of this invention. For example, such constructs may be characterized by an ATG start codon fused directly to the nucleotides encoding the first amino acid of the LFA-3 polypeptide. This construction may produce an f-Met polypeptide. However, it will be understood that the initial methionine may be cleaved during expression in a transformed host or may be subsequently removed. Alternatively, a DNA sequence encoding a bacterial or eukaryotic signal sequence may be fused to the 5' end of a DNA sequence encoding an LFA-3 polypeptide of this invention. This would allow the expressed product to be either secreted or targeted to a specific subcellular compartment within the host cell. Most signal sequences are removed by the host cell after performing their targeting function, thus obviating the need for removal after purification of the desired polypeptide. Many signal sequences, as well as the DNA sequences encoding them, are known in the art. The fusion protein of such signal sequence DNA to and in frame with the sequence encoding an LFA-3 polypeptide

of this invention can be achieved by standard molecular biology techniques.

Alternatively, a DNA sequence encoding an LFA-3 polypeptide of this invention may be expressed as a fusion protein by in-frame ligation to a second DNA sequence encoding a host cell polypeptide. The expression of a fusion protein may afford several advantages, such as increased resistance to host cell degradation, ease of identification based upon the activity or antigenicity of the host cell polypeptide, and ease of purification, based upon the physical or immunological properties of the host cell polypeptide.

This invention also relates to hosts transformed with the recombinant DNA molecules described above. Useful hosts which may be transformed with these recombinant DNA molecules and which may be employed to express the LFA-3 polypeptides of this invention may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, e.g., *E. coli* SG-936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DH1, *E. coli* DH5-alpha, *E. coli* MRC1; strains of *Pseudomonas*; strains of *Bacillus*, such as *Bacillus subtilis*; strains of *Streptomyces*; strains of *Saccharomyces*; animal cells, such as COS cells, CHO cells, BHK cells, human tissue cells; insect cells (e.g., *Spodoptera frugiperda* (SF9)); and plant cells in tissue culture. The preferred host for polypeptides claimed herein is CHO cells.

It will be appreciated that not all host/expression vector combinations will function with equal efficiency in expressing DNA sequences encoding the LFA-3 polypeptides of this invention. However, a particular selection of a host-expression vector combination may be made by those of skill in the art after due consideration of the principles set forth herein without departing from the scope of this invention. For example, the selection should be based on a balancing of a number of factors. These factors include, for example, compatibility of the host and vector, toxicity of the polypeptides encoded by the DNA sequence to the host, vector copy number and the ability to control that copy number, the expression of other proteins encoded by the vector, such as antibiotic markers, ease of recovery of the desired protein, expression characteristics of the DNA sequences and the expression control sequences operatively linked to them, biosafety, costs and the folding, form or any other necessary post-expression modifications of the desired polypeptide.

While recombinant DNA techniques are the preferred method of producing the polypeptides of this invention having a sequence of more than 20 amino acids, shorter polypeptides encompassed by this invention having less than about 20 amino acids are preferably produced by conventional chemical synthesis techniques. Synthetically produced polypeptides of this invention can advantageously be obtained in extremely high yields and be easily purified.

In a preferred embodiment of this invention, the polypeptides are synthesized by solution phase or solid phase polypeptide synthesis and, optionally, digested with carboxypeptidase (to remove C-terminal amino acids) or degraded by manual Edman degradation (to remove N-terminal amino acids). Proper folding of the polypeptides may be achieved under oxidative conditions which favor disulfide bridge formation, as described by S. B. H. Kent, "Chemical synthesis of polypeptides and proteins", *Ann. Rev. Biochem.*, 57, pp. 957-89 (1988). Polypeptides produced in this way may then be purified by separation techniques widely known in the art, preferably utilizing reverse phase HPLC. The use of solution phase synthesis advantageously allows for the direct addition of certain derivatized amino acids to the growing polypeptide chain, such as the O-sulfate ester of

tyrosine. This obviates the need for a subsequent derivatization step to modify any residue of the polypeptides of this invention.

The biological activity of the polypeptides of this invention, i.e., their ability to block LFA-3/CD2 interaction, may be assayed using a simple cell binding assay that permits visual (under magnification) evaluation of the binding of LFA-3 polypeptide-expressing cells to CD2-expressing cells. Jurkat cells are preferred as the CD2⁺ substrate (see Examples, *infra*). The binding characteristics of soluble polypeptides according to the invention may be assayed in several known ways, such as by radiolabeling the polypeptide with, e.g., ³⁵S, and then contacting the labeled polypeptide with CD2⁺ cells. Enzymatic labels or rosetting assays such as described by Seed et al. (*Proc. Natl. Acad. Sci. USA*, 84, pp. 3365-69 (1987)) may also be used.

In another embodiment of this invention, fusion proteins and DNA sequences coding for them are provided. These fusion proteins have an amino-terminal region characterized by the amino acid sequence of a CD2-binding polypeptide of this invention and a carboxy-terminal region comprising a domain of a protein or polypeptide other than LFA-3. Such domains include, for example, the Fc region of an immunoglobulin.

In the preferred fusion proteins of this invention, the CD2-binding polypeptides of this invention are fused to at least a portion of the Fc region of an immunoglobulin. In these fusion proteins, the CD2-binding polypeptides form the amino-terminal portion, and the Fc region forms the carboxy terminal portion.

In these fusion proteins, the Fc region is preferably limited to the hinge region and the C_H2 and C_H3 domains. More preferably, the Fc region in the fusion proteins of this invention is limited to a portion of the hinge region, the portion being capable of forming intermolecular disulfide bridges, and the C_H2 and C_H3 domains. See, e.g., FIGS. 12A and 12B.

An example of a useful LFA-3-Ig fusion protein of this invention is LFA3TIP (also referred to as LFA-3(92)IgG), which is secreted into the cell culture medium by COS7 cells containing the expression plasmid pSAB152 (see *infra*). LFA3TIP consists of the amino terminal 92 amino acids of the mature form of LFA-3 fused to a portion of the hinge region and the C_H2 and C_H3 constant domains of human IgG1 (see FIG. 11). The fusion protein, LFA3TIP, contains a functional CD2-binding domain of LFA-3 and a sufficient portion of the Fc region of IgG to be recognized by the Fc binding protein, Protein A. LFA3TIP is able to bind to CD2 and inhibit T-cell activation.

It will be apparent that the polypeptides and fusion proteins of this invention may be used in methods to selectively isolate CD2 or CD2-expressing cells, based on the formation of a complex between the CD2-binding domain of those polypeptides and CD2.

In another embodiment of this invention, the polypeptides and fusion proteins of this invention, such as the LFA-3-Ig fusion proteins, may be used to label cells expressing CD2 or a polypeptide containing the LFA-3 binding domain of CD2. For example, the polypeptides and fusion proteins of this invention may be conjugated to a "reporter molecule" which allows detection of the polypeptides or fusion proteins bound to CD2⁺ cells or polypeptides containing the CD2-binding domain of LFA-3. Owing to the presence of a portion of the immunoglobulin Fc region in the illustrative fusion proteins of this invention, such fusion proteins can be conjugated to the same "reporter molecules" which are

commonly conjugated or bound to immunoglobulins in order to detect immunoglobulins bound to antigens, e.g., ¹²⁵I enzyme-conjugated secondary antibodies directed to the Fc region, or biotin-streptavidin based enzyme conjugated molecules. Accordingly an LFA-3-Ig fusion protein of this invention may be conjugated or bound to such reporter molecules via its Fc carboxy-terminal portion. Furthermore, such reporter molecules may be conjugated or bound to the polypeptides or fusion proteins of this invention before or after the polypeptides or fusion proteins have bound to CD2 or to the LFA-3 binding domain of CD2.

It is a further embodiment of this invention that the polypeptides and fusion proteins of this invention can also be used in diagnostic applications to detect the presence or indicate the absence of CD2 or cells or molecules containing the LFA-3-binding domain of CD2.

The polypeptides and fusion proteins of this invention can also be used in therapeutic compositions to inhibit formation of the CD2/LFA-3 complex, when such formation contributes to a pathological state. Alternatively, they may be used therapeutically to mimic the role of LFA-3 in initiating one or more of the T-lymphocyte functional responses dependent on the formation of the CD2/LFA-3 complex (see, e.g., Dustin et al., *J. Exp. Med.*, 165, pp. 677-92 (1987); Springer et al., *Ann. Rev. Immunol.*, 5, pp. 223-52 (1987)). For example, the polypeptides and fusion proteins of this invention may be used in the treatment of acute and chronic inflammation, autoimmune diseases, and for immunomodulation, including treatment of such diseases as systemic lupus erythematosus or lupus vulgaris, rheumatoid arthritis and thyroiditis. Furthermore, the polypeptides and fusion proteins of this invention may be used to inhibit T-cell activation or to inhibit proliferation of peripheral blood lymphocytes.

In these respects, it is recognized that molecules involved in cell-cell adhesion are generally more effective in eliciting a particular response from a cell when the molecules are present in a multimeric form as opposed to a monomeric form of the same protein. Multimeric forms of cell surface adhesion proteins appear to more closely mimic the typical situation in vivo where, e.g., an effector cell will exhibit hundreds or thousands of copies of a particular adhesion protein on its surface which then bind to the many copies of its ligand on a target cell. When many surface molecules become involved in binding, they may act synergistically, so that the affinity of one cell for another is greater than the mere sum of the binding affinities of the individual molecules. Accordingly, an important aspect of this invention concerns the preparation and use of multimeric forms of the CD2-binding polypeptides disclosed herein.

A variety of methods are known in the art to form multimeric forms of protein monomers. Such methods include using crosslinking agents, e.g., glutaraldehyde (e.g., Reichlin, *Methods Enzymol.*, 70, pp. 159-65 (1980)). If thiol residues are present on a polypeptide or polypeptides, such groups may be oxidized to form intermolecular disulfide bonds to achieve multimeric forms of the polypeptide or polypeptides. Thiol residues or thiol-reactive groups may be introduced into a polypeptide using iminothiolane or heterobifunctional cross-linkers, such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), which contains an amine-reactive group and a thiol-reactive group. Coupling of the proteins may then be accomplished through disulfide bonds formed either directly or through homobifunctional cross-linking agents (see, e.g., Srinivasachar et al., *Biochem.*, 28, pp. 2501-09 (1989); Ramakrishnan et al., *Cancer Res.*, 44, pp. 201-08 (1984); Lambert et al., *J. Biol. Chem.*, 260, pp.

12035-41 (1985)). The effectiveness of disulfide bond formation between molecules would of course be limited to the number of thiols available on the polypeptide (naturally occurring or introduced by derivatization as above) and whether such disulfide bond formation adversely affected the affinity of the resulting multimeric form.

If polypeptides or proteins possess carbohydrate groups, such as in glycoproteins, the sugar moieties of such groups may be used in reactions to link one molecule with another (e.g., Liao et al., *J. Biol. Chem.*, 248, pp. 8247-53 (1973); Moroney et al., *Biochem.*, 26, pp. S390-98 (1987)).

Other multimeric forms of the CD2-binding polypeptides of this invention may be produced by attaching a phosphatidylinositol ("PI") linkage sequence, e.g., as described in PCT patent application PCT/US90/01859, or sequences of C4 binding protein, e.g., as described in PCT patent application PCT/US91/00567. The hydrophobic PI anchor may be used to form micelles exhibiting a plurality of active CD2-binding domains or multimeric forms of the PI-linked LFA-3, in which a plurality of monomers are associated with one another by hydrophobic interactions between the PI linkage sequences of each monomer (in the absence of added lipids or membrane). In this latter case, multimeric PI-linked LFA-3 molecules are usually octamers; though other polymeric forms have been observed (e.g., 6-12 associated monomers). Alternatively, attaching a segment of DNA encoding the PI linkage sequence downstream of a DNA insert encoding a polypeptide of the invention, and transfecting suitable mammalian host cells with this construct, will provide a culture of cells exhibiting numerous copies of the CD2-binding polypeptide on their surfaces (attached by a PI anchor).

Alternatively, multiple copies of monomers of polypeptides and fusion proteins of this invention may be bound to another molecule or substrate or particle. As in the case of the binding of LF08 to Affigel-10 beads (see infra), the formation and use of molecules, compounds or particles comprising multiple CD2-binding domains are within the scope of this invention.

In addition, this invention also includes multimeric forms of LFA-3-Ig fusion proteins. Such multimers may be generated by using those Fc regions, or portions thereof, of Ig molecules which are usually multivalent, e.g., IgM pentamers and IgA dimers. It is of course understood that a J chain polypeptide may be necessary to form and stabilize IgM pentamers and IgA dimers. Alternatively, multimers of LFA-3-Ig fusion proteins may be formed by using a protein with an affinity to the Fc region of Ig molecules, such as Protein A. For example, a plurality of LFA-3-Ig fusion proteins, such as LFA3TIP, may be bound to Protein A-agarose beads to form agarose beads whose surfaces are covered with multiple functional CD2-binding domains of the attached LFA-3-Ig fusion proteins.

In another embodiment, this invention provides multimeric proteins capable of binding to CD2, which comprise (a) two or more of the CD2-binding polypeptides described herein, (b) two or more of the CD2-binding fusion proteins described herein, or (c) one or more of the CD2-binding polypeptides and one or more of the CD2-binding fusion proteins.

The polypeptides of this invention may also be used to design low molecular weight (i.e., usually monomeric molecular weights of less than 1000 daltons) nonpeptidyl or only partial peptidyl CD2-binding molecules useful to inhibit cell-cell adhesion via formation of the CD2/LFA-3 complex. Based on the polypeptides provided herein, such

small molecules may be designed which possess the ability to bind CD2 and may consist of moieties other than amino acids and may be produced entirely through synthetic methods. The use of such small molecules as therapeutic or diagnostic agents also falls within the scope of this invention.

Another embodiment of this invention involves the use of the CD2-binding polypeptides and fusion proteins to obtain antibodies recognizing the CD2-binding domain of LFA-3. Both monoclonal antibodies (MAbs) and polyclonal antibodies highly specific to the CD2-binding domain of LFA-3 may be obtained utilizing the polypeptides and fusion proteins of this invention.

Methods for obtaining monoclonal antibodies and polyclonal antiserum to a particular antigen are well known in the art. For producing MAbs, an immortal cell line (typically a myeloma cell line) is fused to lymphocytes (typically splenocytes) from a mammal (e.g., a rabbit) immunized with a particular antigen, i.e., in this case the CD2-binding domain of LFA-3. Such fusions usually generate hybridomas, i.e., immortal clones of hybrid cells which produce antibody molecules specific for a single epitope of the immunizing antigen (see, generally, Kohler et al., *Nature*, 256, pp. 495-97 (1975)). Effective immunization may require that the polypeptide comprising the CD2-binding domain be polymerized or derivatized and mixed with an adjuvant.

It is also necessary to be able to screen the potentially numerous clones of hybridomas generated from the fusions in order to identify those clones which produce antibodies to the CD2-binding domain of LFA-3. For example, such screens may involve assaying the supernatants of cultures of hybridomas for the ability to inhibit CD2-expressing cells from binding to LFA-3 expressing cells. Assays which have been used to screen hybridomas for the production of MAbs to LFA-3 are applicable as primary screens for hybridomas producing MAbs specific for the CD2-binding domain of LFA-3 (see, e.g., Sanchez-Madrid et al., *Proc. Natl. Acad. Sci. USA*, 79, pp. 7489-93 (1982)).

The DNA encoding the CD2-binding polypeptides of this invention and of deletion mutant forms of the LFA-3 gene, such as those described herein, may also be used as plus/minus probes to detect and isolate other DNA sequences encoding the CD2-binding domain of LFA-3.

The deletion mutant forms of the LFA-3 protein, which lack at least amino acids +51 to +60 of mature LFA-3; at least amino acids +41 to +50 of mature LFA-3 or at least amino acids +31 to +40 of mature LFA-3; (and preferably lack all 3 regions) may be used to clear polyclonal antiserum of antibodies which recognize epitopes of LFA-3 other than the epitopes within the CD2-binding domain of LFA-3. Such mutants may be, for example, selected from the group consisting of M57, M55, M56, PIM3 and M100, and combinations thereof. For example, a polyclonal anti-LFA-3 antiserum may be preincubated with a mutant form of the LFA-3 protein encoded by the mutant LFA-3 gene containing the M57 or M100 deletion mutations (FIGS. 1A-1 and 1A-2 and FIGS. 6A and 6B). The polyclonal antibodies recognize and bind LFA-3 epitopes on the mutant LFA-3 protein. However, since the CD2-binding domain is not present, antibodies to this epitope in the polyclonal antiserum will not bind. Precipitation of the complexes that do form will result in an enrichment for any antibodies remaining in the antiserum that are specific for epitopes of the CD2-binding domain.

The CD2-binding domain polypeptides and fusion proteins of this invention may be formulated as pharmaceutical

compositions using conventional methods to prepare pharmaceutically useful compositions and combinations. Such compositions preferably include at least one pharmaceutically acceptable carrier. See, e.g., *Remington's Pharmaceutical Sciences*, (E. W. Martin). Pharmaceutical compositions of the present invention typically contain, in addition to the active polypeptide, a pharmaceutically acceptable buffer, preferably phosphate buffered saline, together with a pharmaceutically acceptable compound for adjusting isotonic pressure, such as sodium chloride, mannitol or sorbitol. The pharmaceutically acceptable compositions and methods of this invention are characterized by pharmaceutically effective amounts of a polypeptide according to the invention.

The pharmaceutical compositions and combinations of this invention which comprise LFA-3-Ig fusion proteins may be used, for example, to inhibit the activation of T-lymphocytes or to inhibit the proliferation of peripheral blood lymphocytes (see *infra*).

The term "combination" as used herein, includes a single dosage form containing at least one polypeptide of this invention and at least one other pharmaceutically active agent, a multiple dosage form wherein the polypeptide and the other active agent are administered separately, but concurrently, or a multiple dosage form wherein the two components are administered separately but sequentially. Alternatively, the polypeptides of this invention and the other active agent may be in the form of a single conjugated molecule. Conjugation of the two components may be achieved by standard cross-linking techniques well known in the art. A single molecule may also take the form of a recombinant fusion protein.

The pharmaceutical compositions and combinations of this invention may be administered to a patient in any pharmaceutically acceptable dosage form, including, but not limited to, those which may be administered to a patient intravenously as a bolus or by continued infusion, intramuscularly, subcutaneously, intracutaneously, intra-articularly, orally or parenterally.

Methods for determining pharmaceutically effective dosages are known to those skilled in the art. The dosage and dose rate will depend on a variety of factors such as the specific composition, the object of the treatment, i.e., therapy or prophylaxis, method of administration, and the judgment of the treating physician.

This invention also relates to the bioanalytic use of CD2-binding polypeptides and fusion proteins, or compositions containing them, for determining the concentration of CD2 proteins or the detection of CD2-expressing cells in a biological sample. These polypeptides and compositions may be used in a manner similar to that of reagents employed in conventional assays. In addition, the polypeptides of this invention may be utilized in diagnostic kits designed to detect the presence and measure the concentration of CD2 or CD2-expressing cells.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of this invention.

EXAMPLE 1

Preparation of Deletion Mutations In The LFA-3 Gene

In order to map the CD2-binding domain on the LFA-3 protein, a series of deletion mutations of the LFA-3 gene were systematically generated using site-specific mutagen-

esis by oligonucleotide heteroduplexing. cDNA encoding transmembrane has been isolated and sequenced (see, Wallner et al., U.S. Pat. No. 4,956,281), and a bacteriophage bearing cDNA coding for transmembrane LFA-3 was deposited with American Type Culture Collection in Rockville, Md. (accession number 75107). A plasmid, pHT16-6, containing cDNA encoding transmembrane LFA-3 was obtained from Biogen, Inc. (Cambridge, Mass.) and used in preparing all the deletion mutations. The construction of plasmid pHT16-6 is described in PCT patent publication WO 88/09820, which is herein incorporated by reference. Plasmid pHT16-6 contains a cDNA insert encoding LFA-3 and contains a unique EcoRI site near the 5' end of the LFA-3 coding sequence, a unique BamHI site adjacent the 5' end of the LFA-3 cDNA, a unique HindIII site adjacent the 3' end of the LFA-3 cDNA, two NotI sites 1112 bp apart and bracketing the LFA-3 cDNA, and a unique ScaI site. The cDNA insert of pHT16-6 containing the entire coding region for transmembrane LFA-3 is shown in FIGS. 1A-1 and 1A-2.

In this procedure, seventeen sequences of the LFA-3 coding sequence, each 30 bp long, were selected for deletion. For each proposed deletion, a 30-base antisense oligonucleotide (30-mer) was synthesized which consisted of the complementary 15 bases immediately upstream of (5' to) the proposed deletion and the complementary 15 bases immediately downstream of (3' to) the proposed deletion. Hybridization of a particular synthetic oligonucleotide to a strand of the LFA-3 DNA resulted in a heteroduplex in which a specific sequence of 30 bp would loop out. By this mutagenesis procedure, some of the replication products of the heteroduplex contained deletion mutations lacking the 30 bp region looped out by heteroduplex formation.

The synthetic oligonucleotides used to generate the seventeen deletion mutants depicted in FIG. 1 are set forth in the following table:

Sequence	Complementary to LFA-3 Nucleotides	Segment Deleted
SEQ ID NO: 13	109-123 + 154-168	M53
SEQ ID NO: 14	139-153 + 184-198	M54
SEQ ID NO: 15	169-183 + 214-228	M55
SEQ ID NO: 16	199-213 + 244-258	M56
SEQ ID NO: 17	229-243 + 274-288	M57
SEQ ID NO: 18	259-273 + 304-318	M58
SEQ ID NO: 19	289-303 + 334-348	M59
SEQ ID NO: 20	319-333 + 364-378	M60
SEQ ID NO: 21	349-363 + 394-408	M61
SEQ ID NO: 22	379-393 + 424-438	M62
SEQ ID NO: 23	409-423 + 454-468	M63
SEQ ID NO: 24	439-453 + 484-498	M64
SEQ ID NO: 25	469-483 + 514-528	M65
SEQ ID NO: 26	499-513 + 544-558	M66
SEQ ID NO: 27	529-543 + 574-588	M90
SEQ ID NO: 28	559-573 + 604-618	M91
SEQ ID NO: 29	589-603 + 634-648	M92.

For each heteroduplex, one sample of plasmid pHT16-6 (100 µg) was digested with HindIII (210 units, New England Biolabs) and either EcoRI (300 units, New England Biolabs) or BamHI (300 units, New England Biolabs). Another sample of pHT16-6 (100 µg) was linearized by digestion with ScaI (300 units, New England Biolabs). Digestion products were electrophoresed on 1 percent (w/v) agarose gels in TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0), and the desired fragment from each digestion was then electroeluted from the gel (Maniatis et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, 1982)). In the case of the ScaI digestion, the linearized pHT16-6 was

electroeluted from the gel; in the case of the HindIII/EcoRI or HindIII/BamHI digestions of pHT16-6, the larger of two restriction fragments produced in each case was electroeluted.

A sample of the ScaI linearized pHT16-6 DNA (100 ng) was mixed with a sample of the isolated HindIII/EcoRI or HindIII/BamHI restriction fragment (100 ng) and with 8 picomoles of one of the seventeen antisense oligonucleotides previously phosphorylated with ATP (Maniatis et al., supra). Referring to FIGS. 1A-1, 1A-2 and 1B, since the EcoRI restriction site is located in the M56 region, the HindIII/EcoRI large restriction fragment could not be used to generate deletions of regions M53, M54, M55 and M56. Thus, the HindIII/BamHI large restriction fragment was used in the procedure to generate these four deletions.

In order to form heteroduplexes, a synthetic oligonucleotide, ScaI-linearized pHT16-6, and either HindIII/EcoRI or HindIII/BamHI large fragment of pHT16-6 were mixed in 100 mM NaCl, 80 mM MgCl₂, 6.5 mM Tris/Cl, pH 7.6 and boiled for 3 minutes to denature. The denatured DNA was allowed to anneal at 37° C. for 1 hour, then at 4° C. for 1 hour, and finally at 0° C. for 10 minutes.

After annealing, the gaps in the heteroduplexes were filled in and the fragments ligated in a single reaction mixture by adding to the annealed DNA the following constituents to give the indicated final concentrations or amounts: ATP (20 mM); dATP, dTTP, dGTP, dCTP (each at 10 mM); Klenow (large fragment of DNA Polymerase I, 2.5 units, New England Biolabs); T4 DNA ligase (200 units, New England Biolabs). The mixtures were then incubated overnight at 15° C.

After incubation, half of each fill-in/ligation mix was used to transform 100 µl of *E.coli* JA221 (Maniatis et al., supra). Colonies of transformed cells were selected on LB plates supplemented with ampicillin (50 µg/ml) (Maniatis et al., supra).

Colonies of transformants were screened for the presence of a particular deletion by colony hybridization using the particular oligonucleotide used in the heteroduplex to generate the deletion as a probe.

For use as probes, a sample of each oligonucleotide (80 pmol) was labeled using ³²P-ATP by standard procedures. The labeled oligonucleotides were precipitated with 2M ammonium acetate (Maniatis et al., supra). Labeled probe was added to the hybridization buffer at a final activity of approximately 5×10⁵ CPM/ml of hybridization mixture. Hybridizations were carried out on nitrocellulose filters at 60° C. for about 8 hours. After washing the filters in 0.5× SSC buffer at 60° C., autoradiography was performed and positive colonies scored (Maniatis et al., supra). The generation of the desired deletion mutations was confirmed by DNA sequence analysis (Maxam and Gilbert, in *Methods in Enzymology* (Grossman and Moldave, eds.), 65, pp. 499-560 (1980)) of plasmid DNA in colonies scoring positive according to hybridization results.

These procedures produced plasmids, each containing one of the 30 bp deleted sequences listed above and depicted in FIGS. 1A-1, 1A-2 and 1B. The plasmid containing the M57 deletion in the LFA-3 coding sequence was designated plasmid pLFA3M57-4A.

EXAMPLE 2

Construction of Recombinant Expression Plasmids

Plasmids prepared according to Example 1 with successfully deleted 30 bp segments from the LFA-3 coding

sequence were used for construction of expression vectors. Aliquots of 100 µg of plasmids having deleted segments (see FIGS. 1A-1, 1A-2 and 1B) M54, M55, M56, M57, M58, M63 and M65, respectively, were digested with NotI endonuclease (100 units, New England Biolabs, Beverly, Mass.) 5 For M57, M63 and M65, the 5' overhangs were filled in using a standard Klenow (New England Biolabs) reaction mix (Maniatis et al., supra) to generate blunt ended restriction fragments. The fragments were purified from 0.7% TAE agarose gels by electroelution.

The gel-purified, blunt-ended, NotI fragments were then individually cloned into the SmaI site of the eukaryotic expression vector pBG368PY (Dailey et al., *J. Virol.*, 54, pp. 739-49 (1985)) using T4 ligase at 15° C. overnight. NotI fragments of M54, M55, M56, and M58 were gel purified as described above and ligated to NotI-digest expression vector, PMDR902. The resulting ligations were used to transform *E. coli* JA221 and positive colonies of transformants containing the recombinant plasmids carrying the desired inserts were identified by colony hybridization, as described above. The orientation of the M57, M63 and M65 inserts in the vector was confirmed by EcoRI digestion, generating 6600, 4000 and 200 bp fragments for correctly oriented inserts, which were separated on 0.7% agarose gel. For plasmids containing M54, M55, M56 and M58 inserts, the orientation was determined by PvuII digestion analysis, where the correct orientation generated fragments of 6599 bp, 1185 bp and 506 bp. All fragments were verified by DNA sequencing (Maxam and Gilbert, supra).

The recombinant expression plasmids carrying DNA encoding the M54, M55, M56, and M58 LFA-3 deletion mutations were linearized with AatII and electroporated into Chinese Hamster Ovary (CHO) cells according to the published protocol of J. Barsoum (*DNA Cell Biol.*, 9, pp. 293-300 (1990)). Plasmids carrying DNA encoding the M57, M63 and M65 deletion mutations were linearized using NruI and electroporated into CHO cells as above, except that 19 µg of the NruI-digested DNA was ethanol precipitated overnight at -20° C. with 1 µg of EcoRI-digested SV2 DNA (DHFR⁺) and 380 µg of sonically disrupted salmon sperm DNA. CHO cells (1×10⁷) were electroporated with the coprecipitated DNA at 280 volts using a BioRad Gene Pulser.

After electroporation, cells were seeded into two 100 mm plates in alpha⁺ MEM, 10% fetal calf serum (FCS), 4 mM L-glutamine, penicillin/streptomycin, then incubated for 48 hours at 37° C. The cells in the plates were then divided into five 100 mm plates containing alpha⁻ MEM, 10% FCS, 4 mM L-glutamine, penicillin/streptomycin, and incubated for five days at 37° C. The cells were then fed with alpha⁻ Complete Medium supplemented with 200 nM methotrexate for M57, M63 and M65, and 50 nM methotrexate for M54, M55, M56 and M58. Each plate was grown to 90% confluency then assayed by FACS (Fluorescence-Activated Cell Sorter) to determine whether any of the CHO cultures transfected with the LFA-3 deletion mutations expressed the mutant forms of LFA-3.

EXAMPLE 3

FACS Analysis of Transfected Cells

Transfected CHO cells, prepared in Example 2 and grown in the presence of methotrexate to 90% confluency, were rinsed twice with HANKS BSS medium (Ca⁺⁺ and Mg⁺⁺ free, pH 7.0) and removed with HANKS (Ca⁺⁺ and Mg⁺⁺ free, 5 mM EDTA, pH 7.0) medium. Approximately 2×10⁵

cells were resuspended in 100 µl of a solution of cold (0° C.) wash buffer (PBS, 0.1% NaN₃, 0.5% bovine serum albumin (BSA), pH 7.2), and a primary antibody was added. The primary antibody was either anti-LFA-3 MAb TS2/9 ascites fluid (see, e.g., Sanchez-Madrid et al., *Proc. Natl. Acad. Sci. USA*, 79, pp. 7489-93 (1982), obtained from Dana Farber Cancer Inst., Boston, Mass.); the anti-LFA-3 MAb 7A6 (obtained from Biogen, Inc., Cambridge, Mass.; 1.6 µg/ml); or anti-LFA-3 polyclonal rabbit antiserum 202 (obtained from Biogen, Inc.). All the primary antibodies are known to block CD2 adhesion to LFA-3. The amount of primary antibody used was typically between 1 and 2 µl. Cells were incubated at 0° C. for 45 minutes and washed twice with wash buffer.

A fluorescein-labeled secondary antibody was then added and the mixture incubated for 30 minutes at 0° C. For analysis with the monoclonal antibodies, the secondary antibody was fluorescein (DTAF)-conjugated affinity purified goat anti-mouse F(ab)₂ IgG (H+L) (Jackson Immuno-research Laboratories, Inc., West Grove, Calif.); for use with the polyclonal rabbit antiserum 202, the secondary antibody was fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (H+L) (Fisher Biotech, Pittsburgh, Pa.).

After incubation with the secondary antibody, cells were overlaid with 300 µl FCS, washed twice in wash buffer, resuspended in 300 µl × PBS and transferred to Falcon 2052 tubes to be read on the cell sorter.

Referring to FIGS. 2A and 2B, the FACS analyses indicated that cells transfected with expression vectors containing deletion mutations M57 (FIGS. 2A and 2B), M65 (FIGS. 2C and 2D), M63 (FIGS. 2E and 2F), M54 (FIGS. 2G and 2H), M55 (FIGS. 2I and 2J), M56 (FIGS. 2K and 2L), and M58 (FIGS. 2M and 2N) expressed a surface protein that was recognized by the anti-LFA-3 polyclonal antiserum 202 (FIGS. 2A, 2C, 2E, 2G, 2I, 2K, 2M). However, the anti-LFA-3 MAbs TS2/9 and 7A6 bound only to transfectants carrying the M65 and M63 deletion mutants and not to transfectants lacking the M57, M54, M55, M56, or M58 regions (compare FIGS. 2B, 2H, 2J, 2L, 2N with 2D, 2F). These results indicated that the M54, M55, M56, M57 and M58 segments, which were deleted from the LFA-3 protein in, respectively, the deletion mutations M54, M55, M56, M57 and M58 (see FIGS. 1A-1, 1A-2, and 1B), were important in CD2 recognition of the LFA-3 molecule.

EXAMPLE 4

Jurkat Cell Binding Assay

CHO cells transfected with the M57 deletion mutation ("M57/CHO" cells), CHO cells transfected with plasmid p24 (see, Wallner et al., PCT patent application WO 90/02181) expressing PI-linked LFA-3 ("P24/CHO" cells, positive control), and normal CHO cells (negative control) were tested for the ability to bind Jurkat cells expressing CD2.

M57/CHO cells, P24/CHO cells and CHO cells (1×10⁵) were added to separate wells of a 6-well plate (Corning) and washed twice with RPMI Complete Medium. Jurkat cells (CD2⁺), obtained as a gift from Dr. Timothy Springer (Dana Farber Cancer Inst., Boston, Mass.) were washed three times with a solution of RPMI, 10% FCS, 4 mM L-glutamine, and then 5×10⁶ cells were added to each well and incubated for 4 hours at 0° C. Cells were then washed gently three times with RPMI and examined under the microscope at 40× and 100× magnification for cell-cell binding.

Referring to FIGS. 3A to 3C, whereas positive control cells expressing PI-linked LFA-3 bound Jurkat cells expressing CD2 (FIG. 3C), M57/CHO cells failed to bind Jurkat cells (FIG. 3B), as did nontransfected (LFA-3⁻) CHO cells (FIG. 3A).

EXAMPLE 5

Northern Blot Analysis of mRNA from M57/CHO Cells

The mRNA of M57/CHO cells was analyzed by Northern blot as a further confirmation that the failure of the monoclonal antibodies, i.e., MAbs TS2/9 and 7A6, to bind to the M57/CHO cells was due to the surface LFA-3 mutant lacking the M57 CD2-binding region rather than to inefficient expression of the mutant LFA-3 gene.

RNA from about 1×10^7 M57/CHO cells on a 100 mm plate was isolated as follows: Growth media were removed and 2 ml extraction buffer (50 mM Tris-Cl (pH 7.5), 1% SDS, 5 mM EDTA, proteinase K added to 100 μ g/ml immediately prior to use) was added. The plate was incubated 20 minutes at 37° C. with gentle shaking. A viscous cell lysate developed and was collected in a 50 ml test tube. An equal volume of phenol:chloroform:ether (50:50:1) was added and the mixture vortexed briefly. The mixture was homogenized on a Polytron mixer (Kinematica, Switzerland) for 15 seconds at highest speed to shear the DNA. The mixture was poured into a 15 ml Corex tube and centrifuged at 10,000 rpm, 10 minutes at 4° C.

After centrifuging, the aqueous phase was collected and NaCl was added to 0.25M, followed by 1 volume of isopropanol. This mixture was placed on dry ice for 10 minutes, defrosted and centrifuged 15 minutes at 10,000 rpm and 4° C.

The pellet was resuspended in 2.9 ml distilled water and vortexed. 0.9 ml of 12M LiCl was added (to 2.8M) and the suspension allowed to stand at least four hours at 4° C. 5S RNA and DNA remained in solution; other RNA fractions precipitated. This solution was centrifuged for at least 15 minutes at 10,000 rpm at 4° C.

The pellet was resuspended in 360 μ l distilled water and transferred to an Eppendorf tube. 40 μ l 3M NaAc (pH 5.2) and 1 ml ethanol were added, then the mixture was cooled at -70° C. for 5 minutes, cooled at -20° C. for 15 minutes, centrifuged, rinsed with ethanol, and resuspended in 400 μ l 0.3M NaAc. Ethanol (1 ml) was added and the precipitation was repeated. The RNA recovered after centrifuging was resuspended 100 μ l distilled water.

10 μ g of the RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to a GeneScreen nitrocellulose membrane (New England Nuclear) and hybridized to a ³²P-labeled probe. As a control, RNA extracted from the cell line M16.3/CHO (expressing recombinant soluble LFA-3, obtained from Biogen, Inc., Cambridge, Mass.) was analyzed on the same Northern blot. The Northern blot analysis (FIG. 4) indicated efficient synthesis of the M57 RNA in the M57/CHO cells.

EXAMPLE 6

Immunoprecipitation of LFA-3 Deletion Mutant

To verify the loss of specific antibody binding domains in the mutant LFA-3 expressed on the surface of M57/CHO cells, M57/CHO transfectants, untransfected CHO cells, and P24/CHO transfectants (which express PI-linked LFA-3)

were surface labeled with ¹²⁵I, and each of the three primary antibody preparations (i.e., polyclonal antiserum 202, MAb TS2/9 and MAb 7A6) were used to immunoprecipitate LFA-3 after detergent disruption of the surface membrane.

About 10^7 (each) of M57/CHO cells, normal CHO cells and P24/CHO cells suspended in PBS (i.e., PBS without Ca⁺⁺ and Mg⁺⁺) supplemented with 5 mM EDTA were washed three times with PBS and resuspended in 0.5 ml PBS. Washed cells were added to glass tubes (12x75 mm²) previously coated with a solution of 50 μ g 1,3,4,6-tetrachloro-3 α , 6 α -diphenylglycoluril (Iodo-Gen, Pierce Bio-Research, Rockford, Ill.) in 100 μ l CHCl₃ and dried in nitrogen. Just before use, the tubes were rinsed with PBS, and then ¹²³Iodine (1 mCi) was added and the tubes incubated on ice for 30 minutes, with swirling every 10 minutes.

After incubation, the iodinated cells were added to 10 ml alpha⁻ MEM, 10% FCS, 4 mM L-glutamine. The cells were spun down, washed twice with 5 ml of the same medium, and then resuspended in 1 ml PBS and centrifuged again. The washed cells were then lysed by resuspension in 1 ml DOC buffer (20 mM Tris/Cl, pH 7.3; 50 mM NaCl, 0.5% deoxycholate (DOC); 0.5% NP40) containing 20 μ l of protease inhibitor PMSF (Sigma Chemical Co., St. Louis, Mo., 17 mg/ml in ethanol), followed by incubation for 30 minutes on ice. The lysate was then microcentrifuged for 10 minutes at 4° C., and the supernatants containing the ¹²⁵I-labeled surface molecules removed.

Lysates from the foregoing iodinations were precleared by contacting with 50 μ l of protein A-Sepharose and incubating on a rocker for 1 hour at room temperature. 100 μ l of lysate was removed for each sample and mixed with 300 μ l of DOC buffer. This mixture was centrifuged and the resulting cleared supernatant removed to a new tube.

Primary antibody was added to samples of each of the cleared supernatants: Polyclonal anti-LFA-3 antiserum 202 was used at a final concentration 40 μ g/ml; MAbs TS2/9, 7A6 and a control monoclonal antibody, MOPC-21 (IgG1, not specific to LFA-3) were used at 5 μ g/ml. 100 μ l DOC and 2 μ l protease inhibitor PMSF were added, and the mixtures incubated on a rocker for 2 hours at room temperature 25° C. or overnight at 4° C.

After incubation, 30 μ l of protein A-Sepharose (for polyclonal antiserum 202 samples) or 15 μ l of anti-mouse IgG-agarose (Sigma Chemical Co.) (for MAb samples) was added to the samples, followed by incubation on a rocker for 2 hours at room temperature. The mixtures were then centrifuged for 2 minutes, and the supernatants carefully removed and discarded. 1 ml of DOC buffer was then added, and the pellets resuspended by vortexing. The resuspended material was then washed 3 times with DOC buffer.

To prepare the samples for analysis by polyacrylamide gel electrophoresis, 30 μ l of SDS sample buffer was added, the sample heated at 65° C. for 15 minutes. The sample was then centrifuged for 2 minutes, and the supernatant removed and saved for electrophoresis. 15 μ l of the supernatant was run on a denaturing 10-20% gradient polyacrylamide gel (Daiichi system, Enprotech), and autoradiography performed to identify immunoprecipitated surface proteins.

FIG. 5 is an autoradiograph of an SDS polyacrylamide gel showing antibody-precipitated LFA-3 surface proteins from M57/CHO, normal CHO, and P24/CHO cells. This gel revealed that TS2/9, 7A6 and the polyclonal antiserum 202 precipitated LFA-3 from the CHO cells expressing the PI-linked LFA-3 (lanes 9, 10, 11). In contrast, only polyclonal antiserum 202 precipitated the LFA-3 surface protein from M57/CHO cells expressing the deletion mutant M57 LFA-3 gene (lane 4).

EXAMPLES 7 and 8

The 10-amino acid region removed in the M57 deletion mutant (see FIGS. 1A-1, 1A-2 and 1B) is adjacent to but does not contain a N-linked glycosylation site. To investigate whether the region of the M57 deletion is involved directly in CD2-binding or indirectly disrupts CD2-binding by causing a conformational change in the CD2-binding domain of LFA-3, two additional experiments were performed.

First, three large deletion mutants were generated, each of which lacked a span of 59 amino acids compared to the native LFA-3 molecule. The regions deleted are shown by underlining in FIG. 6 and were designated M100, M101 and M102. The object of this experiment was to determine whether the conformational changes inherent in the large M101 and M102 deletions would alter recognition of the M57 region (see FIG. 1) and to demonstrate that the entire CD2 binding domain was encompassed in the M100 region.

Second, a synthetic oligopeptide, LF08, having the amino acid sequence (SEQ ID NO:7) Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr (amino acids +50+65 of mature LFA-3 (see FIGS. 1A-1, 1A-2 and 1B)) was isolated and fixed to a solid substrate, then used in a Jurkat cell rosetting assay to determine if CD2-expressing cells would recognize the isolated M57 region of LFA-3 (see FIGS. 1A-1, 1A-2 and 1B).

Preparation of Large Deletion Mutants

FIGS. 6A-1, 6A-2 and 6B depicts the amino acid sequence and nucleotide sequence of transmembrane LFA-3 and shows with underlining the three regions, designated M100, M101 and M102, which were deleted from LFA-3 cDNA to yield genes capable of directing the expression of three large deletion mutants.

Following the procedure used in Example 1, a 30-base antisense oligonucleotide was synthesized which consisted of sequences complementary to the 15 bases of the sense strand upstream of (5' to) and to the 15 bases downstream of (3' to) the desired deletion region. The synthetic oligonucleotides used to generate the large deletion mutations are shown below:

Sequence	Complementary to LFA-3 Nucleotides	Segment Deleted
SEQ ID NO: 30	109-123 + 304-318	M100
SEQ ID NO: 31	289-303 + 484-498	M101
SEQ ID NO: 32	469-483 + 634-648	M102.

Heteroduplexing was carried out as described in Example 1, except that 200 ng of LFA-3 cDNA was used. Colony hybridizations were carried out using 1×10^6 CPM/ml ^{32}P -ATP kinased oligonucleotides at 65° C. and washed with 0.5 \times SSC at 60° C. Positives were screened by EcoRI digestion and isolation of 2900 bp and 500 bp fragments on a 0.7% TAE agarose gel.

Construction of expression vectors was performed by excising the LFA-3 cDNA from deletion mutation plasmids prepared above with NotI. Aliquots of 50 μg of a eukaryotic expression plasmid PMDR902 (obtained from Biogen, Inc., Cambridge, Mass.) were digested with 50 units of NotI, and the isolated NotI fragments (0.06 pmol), containing the deletion mutations M100, M101 and M102, were ligated to 0.02 pmol PMDR902 DNA, and the ligated products were used to transform *E. coli* JA221.

Positive clones were screened by colony hybridization using oligonucleotides 18, 19 and 20, having the sequences of SEQ ID NO:30, SEQ ID NO:31 and SEQ ID NO:32, respectively, as probes. The orientation of the inserts was determined by PvuII restriction analysis, which generated bands of approximately 6599, 1737 and 500 bp on 20.7% TAE agarose gel for correctly oriented constructs. The sequence was confirmed by DNA sequencing as described above. The resulting recombinant expression plasmids containing the M100, M101 and M102 deletion mutations were designated pPMDRM100-4, pPMDRM101-1 and pPMDRM102-8, respectively. The resulting expression plasmids were electroporated into CHO cells as in Example 2, except the cells were split into medium containing 25 nM and 50 nM methotrexate.

The M100/CHO, M101/CHO and M102/CHO transfectants were subjected to a Jurkat cell binding assay, as described in Example 4 above. The results are shown in FIGS. 7A to 7D. M101/CHO cells (FIG. 7C) and M102/CHO cells (FIG. 7D), which express a surface protein having the intact M57 CD2-binding region of LFA-3, showed clumping characteristic of Jurkat/CHO cell binding. CHO control cells (FIG. 7A) and M100/CHO cells (FIG. 7B) showed no such clumping, indicating the absence of a surface structure on the CHO cell surface recognized by Jurkat cells.

Jurkat Binding to Immobilized LF08Peptide

1.4 mg of the synthetic peptide LF08 (SEQ ID NO:7) or control hepatitis B peptide MXC-01 (SEQ ID NO:4) at 2 mg/ml in deionized water was coupled to 100 μl agarose gel beads (Affi-Gel 10, BioRad, Richmond, Calif.) according to the following protocol: 100 μl of Affi-Gel 10 slurry was transferred to a small Buchner funnel and the solvent drained. The beads were washed with three bed volumes of cold (4° C.) deionized water, transferred to 700 μl of the LF08 solution, and then incubated for 4 hours at 4° C. on a rocker. A spectrum taken of the supernatant, before and after coupling of LF08, indicated that a coupling efficiency of 80% had been achieved.

After coupling, unreacted coupling sites were blocked by incubating the LF08 beads or MXC-01 beads with ethanolamine/HCl (93 mM, pH 7.9) overnight at 4° C. with gentle shaking. The blocked beads were then washed three times with PBS supplemented with 0.5M NaCl and three times with PBS supplemented with 10% FCS.

Jurkat cells (2×10^5) were washed twice with RPMI, 4 mM L-glutamine, 10% PBS and combined with 2.5 or 15 μl of LF08/Affi-Gel 10 beads or MXC-01/AffiGel 10 beads to a final volume of 20 μl . The mixture was incubated for 30 or 60 minutes at room temperature. After incubation, the cells were transferred to 200 μl RPMI in a 96-well flat bottom plate (Corning), and observed under a microscope at 40 \times and 100 \times . FIGS. 8A and 8B show characteristic results of contacting the LF08/Affigel beads with Jurkat cells, resulting in binding of coated beads to the surface of Jurkat cells. FIG. 8C shows characteristic results of the MXC-01/Affigel beads failing to bind to the surface of Jurkat cells.

EXAMPLES 9 and 10

A further deletion mutant was prepared which provided a PI-linked surface polypeptide on CHO cells having the N-terminal 89 amino acids of native LFA-3. Referring to FIGS. 9A and 9B, the nucleotides deleted from cDNA encoding PI-linked LFA-3 are shown by the underlined

region, PIM3. The PIM3 nucleotides were deleted using the same heteroduplex deletion mutagenesis strategy described above (Example 1). In this case, a 30-base antisense oligonucleotide (oligonucleotide 320.03), having the 5'-3' sequence (SEQ ID NO:5) TAATGGATTG CTAAGAAAGA ACTTCATGGT, was used for deletion mutagenesis.

Plasmid p24 (100 ng), containing the cDNA encoding PI-linked LFA-3 was digested with NotI, and the large restriction fragment (lacking the PI-linked LFA-3 coding sequence) was isolated. Additional p24 DNA was linearized with ScaI. The large NotI restriction fragment and the ScaI-linearized DNA were denatured, mixed with oligonucleotide 320.03, and allowed to reanneal as-described in Example 1. After annealing, the gaps in the heteroduplexes were filled in, and the fragments ligated as described in Example 1.

The filled-in, ligated heteroduplexes were then transformed into *E. coli* JA221 and the transformants screened by colony hybridization using a ³²P-labeled 320.03 oligonucleotide (1×10⁶ cpm/ml) at 60° C. as described above. The hybridization filters were washed in 0.5× SSC at 65° C. and positive colonies identified by autoradiography. Plasmid DNA was isolated from each of the positive colonies, and digested with NotI. Digestion of one of the plasmids, pPIM3-6, with NotI yielded fragments of 2648 and 640 bp, confirming that pPIM3-6 contained the desired PIM3 deletion in the PI-linked LFA-3 coding sequence.

The 640 bp NotI fragment of pPIM3-6 was cloned into the NotI site of the expression vector PMDR902 (a gift of Dr. M.D. Rosa, Biogen, Inc., Cambridge, Mass.), and used to transform *E. coli* JA221 cells. Transformants were screened by colony hybridization using the ³²P-labeled 320.03 oligonucleotide as probe. Plasmids from positive colonies were then isolated, and the orientation of the DNA insert determined by restriction enzyme analysis using PvuII. Recombinant plasmid pPMDRM3-10 contained PvuII fragments of 6399, 1634 and 495 bp, indicating that this plasmid contained the mutated PI-linked LFA-3 gene in the proper orientation for expression in the PMDR902 vector.

Expression of PIM3 Deletion Mutant

pPMDRM3-10 was electroporated into CHO DHFR⁻ cells and amplified with 25 and 50 nM methotrexate as described above. Cells from plates containing confluent growth were assayed by FACS using MAbs TS2/9 and 7A6 as described above. The PIM3 deletion mutant form of LFA-3 expressed in a cell line designated PIM3.25.2 exhibited epitopes involved with CD2/LFA-3 complex formation.

To determine whether the protein recognized by TS2/9 and 7A6 on PIM3.25.2 cells was PI-linked, the protein expressed on the surface of PIM3.25.2 cells was tested for its susceptibility to release by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC, obtained from Biogen, Inc.). Cells of PIM3.25.2 were grown to 70 percent confluency in two 100 mm culture plates and removed with PBS supplemented with EDTA (5 mM) as described above. One-third of these cells was treated with PI-PLC (1 μl; Biogen, Inc.) at 37° C. for 45 minutes in 100 μl of alpha⁻MEM supplemented with 10% FCS, L-glutamine (2 mM), pen-strep mix (1×), and 25 nM methotrexate. The cells were then centrifuged for 15 seconds in an Eppendorf microcentrifuge and washed two times in FACS buffer (PBS, 0.5% BSA, 0.1% sodium azide, pH 7.2). The washed cells were then resuspended in FACS buffer containing FITC-labeled

goat anti-mouse IgG (1:50 dilution from serum). The cells were then washed twice with FACS buffer, resuspended in 300 μl of PBS and transferred to Falcon 2052 tubes for FACS analysis.

Referring to FIGS. 10A and 10B, cells of PIM3.25.2 clearly express a protein on their cell surface that is recognized by TS2/9 (FIG. 10A, dashed line) and is also susceptible to release from the cell surface by PI-PLC treatment (FIG. 10A, dotted line). Since the PIM3 protein expressed in cells of clone PIM3.25.2 possesses LFA-3 epitopes recognized by MAb TS2/9, which blocks CD2/LFA-3 complex formation, it was concluded that the 89 amino acid N-terminal region of the mature LFA-3 molecule contains the conformational requirements necessary for CD2/LFA-3 complex formation.

To increase expression of the PIM3 mutant LFA-3 protein, the PIM3.25.2 clone was amplified in alpha⁻ MEM supplemented with 10% FCS in 50, 100 and 200 nM of methotrexate. Cells were plated in 96-well microtiter plates at a concentration of 20 cells/ml, grown to confluency (12-17 days) and expanded to 100 mm plates for subsequent FACS analysis as described for PIM3.25.2, except that expression of the PIM3 mutant form of LFA-3 was monitored using the 7A6 MAb (FIG. 10B, dashed line). The results of the FACS analysis indicated that the amplified cell line, PIM3.25.2.100.12, expressed approximately ten-fold higher amounts of the PIM3 mutant form of PI-linked LFA-3. Treatment of the PIM3.25.2.100.12 cells with PI-PLC released about 80% of the PIM3 mutant LFA-3 (FIG. 10B, dotted line).

EXAMPLE 11

Construction of plasmid SAB144

pSAB144 contains the human IgG1 constant region sequences excluding the C_H1 region and the first cysteine of the amino terminal portion of the hinge region. The human IgG1 heavy chain constant region was isolated from a partial Sau3A human fibroblast genomic library (EMBL/5×, a gift of Dr. Mark Pasek). The library DNA was cleaved with HindIII and PvuII. DNA fragments corresponding to approximately 3 kb were ligated to the vector fragment of pSP65 (Promega, Madison, Wis.) generated by cleavage of pSP65 using HindIII and HincII. The resulting plasmid was designated pAB1. Plasmid pAB1 was used as a source of DNA encoding the IgG1 heavy chain constant region.

In order to isolate a cDNA copy of the IgG1 heavy chain region, RNA was prepared from CO87 cells which had been transiently transfected by the plasmid VCAM1-IgG1 (also known as pSAB133). Construction of plasmid VCAM1-IgG1 is described in PCT patent application WO 90/13300. The RNA was reverse transcribed to generate cDNA using reverse transcriptase and random hexamers as the primers. After 30 min. at 42° C., the reverse transcriptase reaction was terminated by incubation of the reaction at 95° C. for 5 min. The cDNA was then amplified by PCR (Polymerase Chain Reaction, see, e.g., Sambrook et al., *Molecular Cloning*, Vol. 3, pp. 14.1-14.35 (Cold Spring Harbor; 1989)) using the following kinased primers: 370-31:

(SEQ ID NO: 34): 5'TCGTC GAC AAA ACT CAC ACA TGC C,
(SEQ ID NO: 35) asp lys thr his thr cys

which contains a SalI site, and

5' GTAAATGAGT GCGGCGGCCG CCAA,

which encodes the carboxy terminal lysine of the IgG1 heavy chain constant region, followed by a NotI site.

The PCR amplified cDNA was purified by agarose gel electrophoresis and glass bead elution for cloning in plasmid pNN03. Plasmid pNN03 was constructed by removing the synthetic polylinker sequence from the commercially available plasmid pUC8 (Pharmacia, Piscataway, N.J.) by restriction endonuclease digestion and replacing the synthetic polylinker sequence with the following novel synthetic sequence (SEQ ID NO:37): GCGGCCGCGG TCCAAC-CACC AATCTCAAAG CTTGGTACCC GGGAAATTCAG ATCTGCAGCA TGCTCGAGCT CTAGATATCG ATTC-CATGGA TCCTCACATC CCAATCCGCG GCCGC.

The purified PCR amplified cDNA fragment was ligated to pNN03 which had been cleaved with EcoRV, dephosphorylated, and purified by low melt agarose gel electrophoresis. The ligation reaction was used to transform *E. coli* JA221 and the resulting colonies were screened for a plasmid containing an insert of approximately 700 bp. The identity of the correct insert was confirmed by DNA sequence analysis, and the plasmid was designated pSAB144.

Construction of Plasmid pSAB149

The plasmid pSAB149 was constructed as follows. The LFA3-encoding plasmid pHT16-6 was subjected to PCR amplification using oligonucleotides 320-04 and 320-05. Oligonucleotide 320-04 includes a NotI site and the nucleotides corresponding to amino acids 1 through 7 of the LFA-3 signal sequence:

(SEQ ID NO: 38) 5' GAGGCGGCCG CC ATG GTT GCT GGG AGC GAC GCG,

(SEQ ID NO: 39) met val ala gly ser asp ala.

Oligonucleotide 320-05 corresponds to the LFA-3 amino acids 86-92 and includes a SalI site (SEQ ID NO:40): 5' AAGTCGACAT AAAGAAAGAA CTTCAT. The amplified DNA fragment was ligated to the vector fragment of pNN03, cleaved by EcoRV.

Construction of pSAB132

pJOD-S (Barsoum, J., *DNA and Cell Biol.*, 9, pp. 293-300 (1990)) was modified to insert a unique NotI site downstream from the adenovirus major late promoter so that NotI fragments could be inserted into the expression vector. pJOD-S was linearized by NotI cleavage of the plasmid DNA. The protruding 5' termini were blunt-ended using Mung Bean nuclease, and the linearized DNA fragment was purified by low melting temperature agarose gel electrophoresis. The DNA fragment was religated using T4 DNA ligase. The ligated molecules were then transformed into *E. coli* JA221. Colonies were screened for the absence of a NotI site. The resulting vector was designated pJOD-S delta NotI. pJOD-8 delta NotI was linearized using SalI and the 5' termini were dephosphorylated using calf alkaline phosphatase. The linearized DNA fragment was purified by low melting temperature agarose gel electrophoresis and ligated in the presence of phosphorylated oligonucleotide ACE175, which has the following sequence: (SEQ ID NO:41) TCGACGCGGC CGCG. The ligation mixture was transformed into *E. coli* JA221, and colonies were screened for the presence of a plasmid having a NotI site. The desired plasmid was named pMDR901.

In order to delete the two SV40 enhancer repeats in the SV40 promoter which controls transcription of the DHFR cDNA, pMDR901 and pJODΔe-tPA (Barsoum, *DNA and Cell Biol.*, 9, pp. 293-300 (1990)) were both cleaved with AatII and DraIII. The 2578 bp AatII-DraIII fragment from pMDR901 and the 5424 bp AatII-DraIII fragment from pJODΔe-tPA were isolated by low melting temperature agarose gel electrophoresis and ligated together. Following transformation into *E. coli* JA221, the resulting plasmid, pMDR902, was isolated. pSAB132 was then formed by eliminating the EcoRI-NotI fragment of pMDR902 containing the adenovirus major late promoter and replacing it with an 839 bp EcoRI-NotI fragment from plasmid pCMV-B (Clontech, Palo Alto, Calif.) containing the human cytomegalovirus immediate early promoter and enhancer.

Construction of pSAB152

pSAB144 was cleaved with SalI and NotI, and the 693 bp fragment isolated. pSAB149 was cleaved with SalI and NotI and the 365 bp fragment was isolated. The two fragments were ligated to pSAB132, which had been cleaved with NotI, and the 5' termini dephosphorylated by calf alkaline phosphatase. The resulting plasmid, pSAB152, contained the DNA sequence encoding the LFA-3 signal sequence, the amino terminal 92 amino acids of mature LFA-3, ten amino acids of the hinge region of IgG1 and the C_{H2} and C_{H3} constant domains of IgG1 (see FIGS. 12A and 12B). *E. coli* JA221 transformed with pSAB152 is deposited with American Type Culture Collection, Rockville, Md. (accession no. 68720).

Construction of pNNM57-14

As mentioned in Example 1, plasmid pLFA3M57-4A contains a DNA sequence encoding LFA-3 with a deletion of the 30 bp M57 region (i.e., nucleotides 244-273 of SEQ ID NO:9). This coding sequence of pLFA3M57-4A was subjected to PCR amplification using oligonucleotide 320-04 (SEQ ID NO:38) and oligonucleotide 320-05 (SEQ ID NO:40) in order to amplify the DNA encoding the signal sequence and the first 82 amino acids of the M57 deletion mutant of LFA-3. The PCR amplified DNA was purified by low melting temperature agarose gel electrophoresis.

The purified, PCR amplified DNA was then ligated into the EcoRV site of plasmid pNN03. The ligated DNA was transformed into *E. coli* JA221, and the resulting transformants screened (by NotI digestion) for a plasmid containing an approximately 300 bp insert. The identity of the desired insert was confirmed by DNA sequence analysis. The desired plasmid containing the approximately 300 bp insert was designated pNNM57-14.

Construction of pM57Ig-5

Plasmid pNNM57-14 was cleaved with NotI and SalI, and the 310 bp fragment isolated by low melting temperature agarose gel electrophoresis. This fragment and the 693 bp SalI-NotI fragment of pSAB144 (see supra) were ligated to NotI-digested, calf alkaline phosphatase-treated pSAB132 (see supra). The ligation mixture was used to transform *E. coli* JA221, and transformant colonies were screened by hybridization using a γ -³²P-ATP labeled oligonucleotide having the sequence of SEQ ID NO:17. The desired orientation of the inserts in pSAB132 was confirmed by the identification of a 6913 and a 2029 bp fragment upon digestion with PvuII. The resulting plasmid was designated pM57Ig-5. DNA sequence analysis confirmed that

pM57Ig-5 contains a DNA sequence encoding the LFA-3 signal sequence, the first 82 amino acids of the M57 deletion mutant of LFA-3, ten amino acids of the hinge region of human IgG1 and the C_H2 and C_H3 constant domains of IgG1.

EXAMPLE 12

Production of LFA3TIP From Transiently Transfected COS7 Cells

To produce the LFA-3-Ig fusion protein, LFA3TIP (also referred to as LFA-3(92)IgG), plasmid pSAB152 DNA was transfected into COS7 cells by electroporation as follows. Four aliquots of 200 µg each of pSAB152 DNA each were ethanol precipitated with 350 µg of sonicated salmon sperm DNA. DNA was pelleted and resuspended in 0.8 ml of 1× HEBS (20 mM Hepes/pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose). COS7 cells from 4 confluent T150 flasks (8×10⁷ cells) were removed by trypsin treatment in DMEM (Gibco, Gaithersburg, Maryland), 10% fetal bovine serum, 4 mM glutamine. Cells from each flask were transferred to individual 15 ml Corning polypropylene tubes and pelleted at 1000 rpm for 4 minutes at room temperature in an IEC centrifuge. Medium was aspirated, and cells resuspended in the DNA-HEBS solution and transferred to a Bio-Rad (Richmond, Calif.) electroporation cuvette. The cuvettes were set in a Bio-Rad Gene Pulser and pulsed at 280 volts and 960 µF capacitance. Cells were resuspended in 10 ml of DMEM medium and pelleted in an IEC centrifuge as described above. All cells were seeded into a 2 liter cell factory in DMEM medium, and culture medium was harvested after 72 hours for purification of LFA3TIP protein.

The concentration of secreted LFA3TIP in transfected COS7 culture medium was determined by ELISA. Fisher 96 flat bottom well plates (Corning 2580) were coated with 50 µl of goat anti-human IgG (H+L) (Jackson Immuno Research, West Grove, Pa., Catalogue No. 109-005-088) diluted at 5µg/ml in 1× PBS. These plates were incubated at 4° C. overnight. Plates were washed the next day 6 times with dH₂O and blocked with 150 µl/well of block buffer (2% normal goat serum in 1× PBS). Buffer was removed after a 2 hour incubation at room temperature and conditioned medium was added at various dilutions. As a control to create a standard curve, a series of dilutions of whole human IgG (Jackson Immuno Research, West Grove, Pa., Catalogue No. 099-000-603) was included on the same plate. After a one hour incubation at room temperature, plates were washed 4 times with 0.01% Tween 20 in 1× PBS, and 50 µl/well of a 1:5000 serum dilution of alkaline phosphatase-conjugated anti-human IgG specific for Fc fragment (Jackson Immuno Research, West Grove, Pa., Catalogue No. 109-055-098) was added to each well. After one hour, plates were washed 6 times with 0.1% Tween 20.

Plates were developed with a solution containing a 1:10 dilution of 10 mM Ca²⁺, Mg²⁺ in 10% diethanolamine, pH 9.6, plus 5 mg/ml 4-nitrophenylphosphate (Boehringer Mannheim Biochemicals, Indianapolis, Ind., catalogue no. 738-352). The reaction was stopped using 3N NaOH and plates read at 405 nM on a Microdevices microplate reader.

Production Of LFA3TIP From A Stably Transformed CHO Cell Line

A recombinant LFA3TIP expression vector was constructed as described below, which can be stably maintained in CHO cells to achieve continuous expression of LFA3TIP.

A NotI fragment containing the LFA3TIP coding sequence of pSAB152 was purified by low melting temperature agarose gel electrophoresis. The fragment was ligated into the NotI cloning site of the expression plasmid pMDR902 (see Example 11). The resulting ligations were used to transform *E.coli* JA221, and colonies containing the desired, correctly oriented, insert were identified by the presence of 6599, 2058, and 487 bp fragments upon digestion with PvuII. The identity of the correct insert in pMDR902 was confirmed by DNA sequence analysis. The resulting recombinant expression plasmid was designated pMDR(92)Ig-3.

The recombinant expression plasmid pMDR(92)Ig-3 was electroporated into CHO cells according to the published protocol of J. Barsoum (*DNA Cell Biol.*, 9, pp. 293-300 (1990)), with the following changes. 50 µg of AatII-digested plasmid DNA and 350 µg of sonicated salmon sperm DNA were used in the electroporation protocol. In addition, cells were selected in alpha⁻ complete medium supplemented with 25 or 50 nM methotrexate (MTX).

To determine expression levels of secreted LFA3TIP, clones were transferred to a flat bottom 96 well microtiter plate, grown to confluency and assayed by ELISA (see infra). One clone grown in the presence of 25 nM MTX, LFA3TIP.25.11, expressed 5-10 µg of LFA3TIP per ml of culture medium. This clone was expanded and amplified further. Amplification was carried out by seeding 1 cell per well in a 96 well plate with complete medium supplemented with 50 or 100 nM MTX. Clones were grown to confluency and assayed by ELISA. Two clones, LFA3TIP.25.11.50.10A and LFA3TIP.25.11.50.5B, secreted higher levels (i.e., 50-60 µg of LFA3TIP per ml of culture medium) of LFA3TIP and were expanded for further study and purification.

The concentration of secreted LFA3TIP in medium of cultures of CHO cells carrying pMDR(92)Ig-3 (i.e., conditioned medium) was determined by ELISA.

Wells of Immulon 2 plates (Dynatech, Chantilly, Va.) were each coated with anti-LFA3 MAb TS2/9 (gift of Dr. Timothy Springer) with 50 µl of anti-LFA3 TS2/9 MAb diluted to 25 µg/ml in 0.05M sodium carbonate/bicarbonate buffer, pH 9.6, covered with Parafilm, and incubated overnight at room temperature. The next day, plates were washed 6 times with deionized water and blocked with 150 µl/well of a block buffer (5% fetal calf serum in 1× PBS), which had been filtered through a 2 micron filter. The buffer was removed after a 2 hour incubation at room temperature, and conditioned medium was added at various dilutions. As a control to create a standard curve, a series of dilutions of LFA-3 (50 µl per well) was also included. Typically, the well containing the most concentrated LFA-3 standard contained 50 µl of an LFA-3 solution containing 10 ng of LFA-3 in 1× PBS. Block buffer diluted in 1× PBS constituted the negative control. The samples and controls were incubated at room temperature for one hour.

The plates were then washed 6 times with deionized water. Each well was then filled with 50 µl of a 1:5000 dilution of rabbit polyclonal anti-LFA-3 antiserum (e.g., antiserum 202, supra) in 5% fetal calf serum in 1× PBS. After one hour at room temperature, the polyclonal anti-LFA-3 antiserum was removed and the wells were washed 4 times with a solution of 0.05% Tween-20 in 1× PBS. Each well was then filled with 50 µl of HRP-goat anti-rabbit IgG(H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, Pa.; Catalogue No. 111-035-045) at a 1:10,000 dilution in the block buffer containing 2% whole mouse serum (Cappel, Catalogue No. 5011-1380). The plates were then incubated at room temperature for 50-60 minutes.

The HRP-goat anti-rabbit IgG(H+L) solution was removed, and the wells were washed 6 times with 0.05% Tween-20 in 1× PBS. Then, 50 μl of HRP-substrate buffer was added to each well at room temperature. HRP-substrate buffer was prepared as follows: 0.5 ml of 42 mM 3,3',5,5'-tetramethylbenzidine (TMB), (ICN Immunobiologicals, Lisle, S.C., Catalogue No. 980501) in DMSO (Aldrich) was slowly added to 50 ml of substrate buffer (0.1M sodium acetate/citric acid, pH 4.9); followed by addition of 7.3 μl of 30% hydrogen peroxide (Sigma, Catalogue No. H-1009).

The development of a blue color in each well was monitored at 650 nm on a microtiter plate reader. After 7–10 minutes, the development was stopped by the addition of 50 μl of 2N sulfuric acid. The resulting yellow color was read at 450 nm on a microtiter plate reader. A negative control well was used to blank the machine.

Production of M57IgG

A fusion protein consisting of LFA3TIP lacking the ten amino acid M57 region of LFA-3 (M57IgG) was produced substantially as described above for LFA3TIP.

EXAMPLE 13

Purification of LFA3TIP

The COS7(pSAB152) conditioned culture medium was concentrated 10-fold using an AMICON S1Y30 spiral cartridge system (AMICON, Danvers, Mass.). The concentrate was divided into 4×50 ml aliquots and batch-adsorbed with 200 μl per aliquot of Protein-A Sepharose 4B (SIGMA, St. Louis, Mo.) overnight at 4° C. The beads were pelleted at 1000 rpm in a clinical centrifuge, combined, and then packed into a 5 mm diameter column. The resin was washed with 20 ml of PBS supplemented with 500 mM NaCl followed by 20 ml of PBS. The bound proteins were eluted in 150 μl fractions with 50 mM glycine, 250 mM NaCl (pH 3.0) into tubes containing 15 μl of 1M HEPES, pH 7.8. 10 μl of each fraction was run on 12% nonreducing SDS-PAGE gels. The fractions containing the eluted protein were pooled and subjected to Superose-12 gel filtration chromatography developed in PBS (Pharmacia/LKB, Piscataway, N.J.).

Aliquots of 10 μl of the peak protein fractions (determined by measuring the O.D. at 280 nm) were run on 12% SDS-PAGE gels under nonreducing conditions and the fractions containing LFA3TIP with a low concentration of contaminating proteins, as determined on the SDS-PAGE gels and by Western blot analysis (see, e.g., Towbin et al., *Proc. Natl. Acad. Sci. USA*, 74, pp. 4350–54 (1979); *Antibodies: A Laboratory Manual*, pp. 474–510 (Cold Spring Harbor Laboratory, 1988)), were pooled and concentrated in a YM30 Centricon (AMICON, Danvers, Mass.). LFA-3 was detected on Western blots using rabbit anti-LFA-3 polyclonal antiserum 202 (see Example 3, supra) and goat anti-rabbit IgG, which was labeled with horseradish peroxidase (Sigma, St. Louis, Mo.). Blots were developed using the Amersham ECL detection kit (RPN 2106, Amersham, Arlington Heights, Ill.). The final pool was run on 12% SDS-PAGE gels under nonreducing conditions to assess the purity. A UV absorbance spectrum was run to determine the concentration. All preparations purified with Protein A were found to contain a single contaminant, which most likely is bovine IgG, present in the fetal calf serum of the culture medium, and which copurifies with the LFA3TIP.

The purity of LFA3TIP was determined by denaturing and non-denaturing SDS-PAGE (FIG. 13). The results of the SDS-PAGE analysis indicated that LFA3TIP, purified from the culture medium of COS7 cells carrying plasmid pSAB152, is present in the cell culture medium as a dimer of two monomeric LFA-3-Ig fusion proteins, connected by disulfide bonds.

The fact that LFA3TIP is found in the cell culture medium suggests that, as with LFA-3, the signal peptide sequence operates normally, i.e., the signal peptide is cleaved from the amino terminal region of the LFA-3 domain of LFA3TIP in the process of secreting LFA3TIP protein across the cell membrane.

Purification of M57IgG

M57IgG was purified from conditioned culture medium substantially as described above for LFA3TIP. Similar contaminants were observed.

EXAMPLE 14

FACS Analysis of LFA3TIP= Binding

Jurkat cells were transferred into several Eppendorf tubes at 1×10^5 cells/tube. Cells were pelleted for 10 seconds in an Eppendorf microcentrifuge, the medium was aspirated off, and pellets were resuspended in 100 μl of LFA3TIP or anti-CD2 monoclonal antibody (MAb) TS2/18 (Sanchez-Madrid et al., *Proc. Natl. Acad. Sci. USA*, 79, pp. 7489–7493 (1982)), both at 10 μg/ml in FACS buffer (PBS, 0.1% NaN₃, 0.5% BSA pH 7.2). Cells were incubated on ice for 30 minutes, washed twice with FACS buffer, and incubated with 100 μl of the appropriate secondary antibody to detect MABs bound to the Jurkat cells. FITC conjugated goat anti-mouse IgG (H+L) F(ab')₂ (Jackson Immunoresearch) was used to detect the anti-CD2 MAb, TS2/18, and R-Phycoerythrin conjugated AP goat anti-human IgG F(ab')₂ (Jackson Immunoresearch) was used to detect LFA3TIP bound to Jurkat cells. The FITC goat anti-mouse IgG F(ab')₂ was diluted to a 1:50 concentration in FACS buffer, and the R-Phycoerythrin conjugated AP goat anti-human IgG F(ab')₂ was diluted by 1:20 in FACS buffer. Cells were incubated for 30 minutes on ice, washed 2 times and resuspended in 300 μl of 1×PBS and fluorescence detected on a cell sorter.

For competition studies, Jurkat cells were pelleted in Eppendorf tubes as described above and LFA3TIP was added at a concentration of 10 μg/ml in FACS buffer. Cells were incubated for 30 minutes on ice and washed twice with FACS buffer. Pellets were then resuspended in 100 μl of a 1:100 dilution (in FACS buffer) of anti-CD2 MAb-containing ascites fluids: T11₁, T11₂ or T11₃ (all were gifts of Dr. Ellis Reinherz, Dana Farber Cancer Institute, Boston, Mass.). Cells were incubated for 30 minutes on ice, washed twice and resuspended in a 1:20 dilution of R-phycoerythrin conjugated AP goat anti-human IgG F(ab')₂ to detect bound LFA3TIP. After a 30 minute incubation on ice, cells were washed twice and resuspended in 300 μl of 1× PBS and analyzed in a cell sorter.

The TS2/18 monoclonal antibodies and the monoclonal antibodies in ascites fluid T11₁ are specific for the LFA-3 binding domain of CD2. Thus, these antibodies are expected to bind to CD2 molecules on the surface of Jurkat cells and occupy the same site at which LFA3TIP binds. Accordingly, when Jurkat cells were incubated first with an excess of TS2/18 MAb or T11₁ ascites fluid, few if any domains are expected to be available for R-Phycoerythrin conjugated

LFA3TIP to molecules to bind. As shown in FIG. 14A, preincubation of Jurkat cells with either the TS2/18 MAB or T11₁ ascites fluid resulted in few cells being labeled with LFA3TIP. A signal nearly identical to unstained control cells was observed. In contrast, in the absence of either TS2/18 or T11₁ ascites fluid, LFA3TIP bound to Jurkat cells and labeled a significant proportion of the cells (FIG. 14A).

The MAbs present in T11₂ and T11₃ ascites fluid are specific for CD2, but recognize epitopes distinct from the epitope of CD2 involved in CD2/LFA-3 complex formation. As shown in FIG. 14B, preincubation of Jurkat cells with either T11₂ or T11₃ ascites fluid did not prevent LFA3TIP from binding to the Jurkat cells. The results shown in FIG. 14A and 14B indicate that LFA3TIP possesses the functional CD2-binding domain LFA-3.

EXAMPLE 15

Mixed Lymphocyte Reaction

A functional assay for the formation of the CD2/LFA-3 complex and T-cell activation is the mixed lymphocyte reaction ("MLR") (see, e.g., Krensky et al., *J. Immunol.*, 131(2), pp. 611-616 (1983); Bradley, "Mixed Lymphocyte Responses", in *Selected Methods in Cellular Immunology* (Mishell and Shiigi, eds.), pp. 162-164 (W. H. Freeman & Co., San Francisco 1980). This assay is based on the activation of T-lymphocytes in a population of peripheral blood lymphocytes ("PBLs") when the T-lymphocytes ("responder cells") recognize alloantigens in nonproliferating allogenic PBLs ("stimulator cells"). Such activation occurs due to cell to cell adhesion, mediated in part by the binding of CD2 molecules on the T-lymphocytes to the LFA-3 molecules on the allogeneic PBLs.

PBLs were purified from 30 ml blood of two allogeneic human donors over a Ficoll-Paque gradient (Pharmacia, Piscataway, N.J., catalog no. 17-0840-02). Blood was diluted at a 1:2 ratio in RPMI medium and overlaid on a Ficoll gradient at a 2:1 ratio (30 ml blood:15 ml Ficoll). Cells were centrifuged through the gradient in a Sorvall RT6000 centrifuge at 1600 rpm, 20° C., for 30 minutes. The interface containing PBLs was collected into 50 ml polypropylene centrifuge tubes (Corning 25331) and topped with RPMI medium. The cells were then pelleted by centrifugation in a Sorvall RT6000 centrifuge at 1400 rpm, 4° C., for 15 minutes. The cells of the pellets were washed twice with 50 ml RPMI and pelleted as described above. PBLs were resuspended in RPMI complete medium (RPMI, 10% Hyclone FCS, 4 mM glutamine, Pen-Strep) to a concentration of 3×10⁶ cells/ml. To produce stimulator cells, PBLs from one of the donors were irradiated at 2000 rads in a Gammacell Irradiator and brought to a final concentration of 3×10⁶ cells/ml.

Antibodies or LFA3TIP samples were added at the concentrations indicated in FIG. 15 to a polystyrene 96 round bottom well plate (Corning 25850). The highest concentration used for both the MAbs and LFA3TIP was 5 µg/ml. All dilutions and incubations were done in RPMI complete medium. Responder cells (nonirradiated) and irradiated stimulator cells were added to wells at a 1:1 ratio at a final concentration of 1.5×10⁵ cells per well each and plates were incubated for five days at 37° C. On the fifth day, cells were pulsed with 1 µCi [methyl-³H] thymidine (New England Nuclear, Boston, Mass., NET-027) for fifteen hours, and harvested on a Tomtech 96 well harvester. Proliferation of the nonirradiated population of T-lymphocytes was measured by determining incorporation of the ³H-thymidine into

the cells, indicating uptake of the labeled thymidine during T-lymphocyte proliferation. Using this MLR assay, a variety of MAbs and CD2-binding molecules were tested for their effect on CD2/LFA-3 complex formation and T-cell activation.

The anti-LFA-3 MAb 1E6 (produced by hybridoma 1E6-2C12, ATCC accession no. HB 10693) is specific for the CD2-binding domain of LFA-3. As shown in FIG. 15, when 1E6 MAbs were present in the MLR assay, a dose dependent inhibition of T-cell activation was observed. This result is consistent with the view that 1E6 MAbs bind the CD2-binding domain of LFA-3, prevent CD2/LFA-3 complexes from forming between stimulator and responder cells, and thereby inhibit activation of T-cells in the responder cell population.

LFA3TIP exhibited a similar dose dependent inhibition of T-cell activation in the MLR assay (see FIG. 15). To exclude the possibility that the IgG portion of the 1E6 MAbs or LFA3TIP contributed to the observed inhibition of T-cell activation, non-specific human IgG1 (Pierce, Rockford, Ill.) was tested in the MLR assay. As shown in FIG. 15, the nonspecific human IgG did not exhibit the dose dependent inhibition of T-cell activation seen with LFA3TIP or the 1E6 MAbs.

Taken together, these results demonstrate that the inhibitory activity of LFA3TIP on T-cell activation in the MLR assay resides in the LFA-3 domain and not the IgG domain of the fusion protein.

Inhibitory Activity on T-cell Activation of Other Molecules

In addition to LFA3TIP and 1E6 MAbs, the following molecules were also tested for inhibitory activity on T-cell activation in the MLR assay: 7A6 MAb (MAb specific for the CD2-binding domain of LFA-3, produced by hybridoma 7A6-2E5, ATCC accession no. HB 10695), TS2/18 (anti-CD2 MAb, Sanchez-Madrid et al., supra), hIgG (nonspecific total human IgG1), PI-LFA3 (multimeric PI-linked LFA-3 formed by intermolecular hydrophobic interaction at the PI anchor region of each PI-LFA-3 monomer), and a CD4-IgG1 fusion protein (analogous to LFA3TIP, but containing an amino terminal region consisting of a portion of CD4, see, e.g., PCT application WO 89/01940).

A comparison of the inhibitory activity of each of these molecules is shown in the bar graph of FIG. 16. "LFA3IgGA" and "LFA3IgG72A" in FIG. 16 are preparations of LFA3TIP differing in purity, i.e., 75% and 50% respectively. As a control, a "mock" preparation, purified from COS7 cells transfected with vector DNA alone (i.e., no DNA insert encoding LFA3TIP), was added to show that the inhibitory activity on T-cell activation was not derived from a nonspecific inhibitor contained in conditioned COS7 cell culture medium. The mock preparation contains the contaminant which, as discussed in Example 13, copurifies with LFA3TIP and probably is bovine IgG present in the fetal calf serum of the growth medium. All MLR assays were carried out as described above, except that each preparation of molecules was assayed at a concentration of 0.1 µg protein/ml.

MAbs which recognize the CD2-binding domain of LFA-3 (7A6 and 1E6) or the LFA-3 binding domain of CD2 (TS2/18) exhibited significant inhibitory activity on T-cell activation. The ability of LFA3TIP to inhibit T-cell activation increased with purity (compare LFA3IgG72A, 50% purity, with LFA3IgGA, 75% purity). Nonspecific human

IgG1 and the CD4-IgG1 fusion protein failed to inhibit T-cell activation.

The multimeric form of PI-linked LFA-3 also failed to inhibit T-cell activation. Despite the plurality of CD2-binding sites in multimeric PI-linked LFA-3, it appears that this type of multimer neither inhibits nor enhances the human allogeneic MLR.

EXAMPLE 16

PBL Proliferation Assay

Human PBLs were isolated from 20 ml of human donor blood on a Ficoll-Paque gradient, as described above. Purified LFA3TIP was added to 96 well polystyrene round bottom tissue culture plates (Corning 25850) at the concentrations indicated in FIG. 17. PBLs (1×10^5) were then added to each well, and plates were incubated for 3 days at 37° C. All dilutions were carried out in RPMI complete medium (see Example 15). Cells were pulsed after 3 days with 1 μ Ci/well [methyl-³H] thymidine for 15 hours and harvested on a Tomtech 96 well automatic harvester. As controls, PBL proliferation was measured in growth medium alone and in growth medium supplemented with nonspecific human IgG1.

As shown in FIG. 17, the LFA3TIP preparation inhibited proliferation of PBLs. Nonspecific human IgG1 did not inhibit PBL proliferation.

We also assayed the major contaminant in the LFA3TIP preparation from a mock preparation as described in Example 15. As mentioned in Examples 13 and 15 above, this contaminant is probably bovine IgG from the growth medium. FIG. 17 shows that, as with the nonspecific human IgG1, the contaminant also did not inhibit proliferation of PBLs.

PHA and OKT3 Dependent PBL Proliferation

We next assessed the ability of LFA3TIP to inhibit OKT3 dependent (i.e., anti-CD3 dependent) T-cell proliferation. PBLs were isolated as described above from one healthy human donor. For OKT3 dependent PBL proliferation, aliquots of 1×10^5 donor PBLs were incubated for 2 days with 3 ng/ml of OKT3 (Ortho Pharmaceuticals, Raritan, N.J.) alone, and in the presence of LFA3TIP (1 nM and 10 nM) or purified human IgG1 (Pierce Chemical Co., Rockford, Ill.) (1 nM and 10 nM). One aliquot of cells was incubated in medium alone, without OKT3. The cells were then pulsed with [methyl-³H] thymidine as described above. The results of this experiment are shown in FIG. 18.

The results in FIG. 18 show that LFA3TIP was significantly more effective at inhibiting OKT3 dependent PBL proliferation than the control human IgG1 of irrelevant specificity. As with the results in FIG. 17, this indicates that the ability of LFA3TIP to inhibit PBL proliferation resides primarily in the 92 amino acid LFA-3 region, which contains the CD2 binding domain, and not in the IgG portion of LFA3TIP.

We also assessed the ability of LFA3TIP and various other CD2-binding molecules, as well as, the ability of M57IgG, to inhibit phytohemagglutinin (PHA) dependent PBL proliferation, at both suboptimal and optimal concentrations of PHA.

For this assay, 1×10^5 donor cells (human PBLs) were incubated with PHA (Fisher) at 0.1 or 1.0 μ g/ml, either alone or in the presence of PI-linked LFA-3 ("PILFA-3", Wallner

et al., PCT patent application WO 90/02181), monomeric soluble LFA-3 ("mon LFA-3", consisting of amino acids 29-181 of SEA ID NO:10), LFA3TIP, M57IgG (see Example 11), a full-length soluble LFA-3-IgG fusion protein ("FLIgG", consisting of amino acids 29-181 of SEQ ID NO:10 fused to a portion of the hinge region and the C_H2 and C_H3 domains of human IgG1), an anti-CD2 MAb (TS2/18, gift of T. Springer), or an anti-LFA-3 MAb (1E6) produced by hybridoma 1E6-2C12 (ATCC HB 10693). FLIgG was at a concentration of 2 μ g/ml; all the other proteins were at a concentration of 5 μ g/ml. The cells were then pulsed with [methyl-³H] thymidine as described above. The results are displayed in FIG. 19.

Referring to FIG. 19, when PHA was incubated with human PBLs at a suboptimal concentration (i.e., 0.1 μ g/ml), only PI-linked LFA-3 (PILFA-3) was able to stimulate PBL proliferation. M57IgG, monomeric soluble LFA-3 and FLIgG had no effect on suboptimal PHA-stimulated T-cell proliferation. MAb 1E6 and MAb TS2/18 inhibited proliferation 80-90%; LFA3TIP inhibited 70%.

When PHA was present at an optimal level to stimulate PBL proliferation, LFA3TIP, MAb 1E6 and MAb TS2/18 inhibited T-cell proliferation by 41%, 26% and 20%, respectively.

The results in FIG. 19 indicate that the region of LFA-3 defined by the N-terminal 92 amino acids, containing the CD2-binding domain of LFA-3, is capable of inhibiting PHA dependent PBL proliferation, but that the absence of a portion of the CD2-binding domain, as in M57IgG, or the presence of additional LFA-3 amino acid sequence, as in PI-linked LFA-3, monomeric LFA-3 or FLIgG, diminishes the ability of the CD2-binding domain of LFA-3 to inhibit PHA dependent PBL proliferation.

Taken together, the results indicated that inhibition of PBL proliferation by LFA3TIP is due to its LFA-3 domain.

It will be recognized from the foregoing description that a wide variety of CD2-binding polypeptides in addition to those specifically mentioned, as well as fragments and analogues of such polypeptides, will be useful additional embodiments of the present invention. All such embodiments, and other embodiments that are obvious in view of the teachings herein, are specifically contemplated and are included within the scope of the invention as defined in the following claims.

Deposits

Host cells harboring plasmids bearing DNA sequences according to this invention were deposited under the Budapest Treaty with American Type Culture Collection (ATCC), Rockville, Md. (USA) on Mar. 5, 1991. The deposited cultures are identified as follows:

plasmid	host strain	Accession No.	Description: (plasmid contains DNA for:)
pPYM57	<i>E. coli</i> JA221	68545	M57 deletion mutant
pPMDRM54-6	<i>E. coli</i> JA221	68542	M54 deletion mutant
pPMDRM55-9	<i>E. coli</i> JA221	68546	M55 deletion mutant
pPMDRM56-C	<i>E. coli</i> JA221	68551	M56 deletion mutant
pPMDRM58-15	<i>E. coli</i> JA221	68547	M58 deletion mutant

-continued

plasmid	host strain	Accession No.	Description: (plasmid contains DNA for:)
pPYM63-4	<i>E. coli</i> JA221	68544	M63 deletion mutant
pPYM65-8	<i>E. coli</i> JA221	68552	M65 deletion mutant
pPMDRM100-4	<i>E. coli</i> JA221	68550	M100 deletion mutant
pPMDRM101-1	<i>E. coli</i> JA221	68543	M101 deletion mutant
pPMDRM102-8	<i>E. coli</i> JA221	68548	M102 deletion mutant
pPMDRM3-10	<i>E. coli</i> JA221	68549	PIM3 deletion mutant

A host cell harboring a plasmid according to this invention was deposited under the Budapest Treaty with the ATCC on Oct. 1, 1991 is identified as follows:

plasmid	host strain	Accession No.	Description: (plasmid contains)
pSAB152	<i>E. coli</i> JA221	68720	Hybrid DNA sequence encoding LFA-3 signal sequence, N-terminal 92 amino acids of LFA-3, 10 amino acids of IgG1 hinge region, C _H 2 and C _H 3 IgG1 constant domains.

A bacteriophage and a bacterial strain carrying a vector referred to herein were deposited under the Budapest Treaty with In Vitro International, Inc., Linthicum, Md. (USA) on May 28, 1987 and May 24, 1988, respectively, and assigned accession numbers IVI-10133 and IVI-10170, respectively. These deposits were transferred to the ATCC on Jun. 20, 1991. The deposits are identified as:

Designation	Accession No.	Description
λHT16[λgt10/LFA-3]	75107	Contain
<i>E. coli</i> , BG8	68791	DNA encoding full length transmembrane LFA-3

The following hybridoma cell lines referred to herein were deposited under the Budapest Treaty with the ATCC on Mar. 5, 1991:

Hybridoma	Accession No.	Antibody
7A6-2E5	HB 10695	7A6
1E6-2C12	HB 10693	1E6

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic embodiments can be altered to provide other embodiments that utilize the compositions and processes of this invention. Therefore, it will be appreciated that the scope of this invention includes all alternative embodiments and variations which are defined in the foregoing specification and by the claims appended hereto; and the invention is not to be limited by the specific embodiments that have been presented herein by way of example.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 43

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Arg Val Tyr Lcu Asp Thr Val Scr Gly
 1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

-continued

Pro	Leu	Lys	Glu	Val	Leu	Trp	Lys	Lys	Gln	Lys	Asp	Lys	Val	Ala	Glu
	50					55					60				
Leu	Glu	Asn	Ser	Glu	Phe	Arg	Ala	Phe	Ser	Ser	Phe	Lys			
65					70					75					

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATAGGGTTT ATTTAGACAC TGTGTCAGGT

30

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 16 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys	Asn	Arg	Val	Tyr	Leu	Asp	Thr	Val	Ser	Gly	Ser	Leu	Thr	Ile	Tyr
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TAATGGATTG CTAAGAAAGA ACTTCATGGT

30

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1040 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(i x) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 10..759

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGACGAGCC	ATG	GTT	GCT	GGG	AGC	GAC	GCG	GGG	CGG	GCC	CTG	GGG	GTC		
	Met	Val	Ala	Gly	Ser	Asp	Ala	Gly	Arg	Ala	Leu	Gly	Val		
	1				5					10					

48

-continued

CTC Leu	AGC Ser 15	GTG Val	GTC Val	TGC Cys	CTG Leu	CTG Leu 20	CAC His	TGC Cys	TTT Phe	GGT Gly	TTC Phe 25	ATC Ile	AGC Ser	TGT Cys	TTT Phe	96
TCC Ser 30	CAA Gln	CAA Gln	ATA Ile	TAT Tyr	GGT Gly 35	GTT Val	GTG Val	TAT Tyr	GGG Gly 40	AAT Asn	GTA Val	ACT Thr	TTC Phe	CAT His	GTA Val 45	144
CCA Pro	AGC Ser	AAT Asn	GTG Val	CCT Pro 50	TTA Leu	AAA Lys	GAG Glu	GTC Val	CTA Leu 55	TGG Trp	AAA Lys	AAA Lys	CAA Gln	AAG Lys 60	GAT Asp	192
AAA Lys	GTT Val	GCA Ala	GAA Glu 65	CTG Leu	GAA Glu	AAT Asn	TCT Ser	GAA Glu 70	TTC Phe	AGA Arg	GCT Ala	TTC Phe	TCA Ser 75	TCT Ser	TTT Phe	240
AAA Lys	AAT Asn	AGG Arg 80	GTT Val	TAT Tyr	TTA Leu	GAC Asp 85	ACT Thr	GTG Val	TCA Ser	GGT Gly	AGC Ser	CTC Leu 90	ACT Thr	ATC Ile	TAC Tyr	288
AAC Asn 95	TTA Leu	ACA Thr	TCA Ser	TCA Ser	GAT Asp	GAA Glu 100	GAT Asp	GAG Glu	TAT Tyr	GAA Glu 105	ATG Met	GAA Glu	TCG Ser	CCA Pro	AAT Asn	336
ATT Ile 110	ACT Thr	GAT Asp	ACC Thr	ATG Met	AAG Lys 115	TTC Phe	TTT Phe	CTT Leu	TAT Tyr	GTG Val 120	CTT Leu	GAG Glu	TCT Ser	CTT Leu	CCA Pro 125	384
TCT Ser	CCC Pro	ACA Thr	CTA Leu	ACT Thr 130	TGT Cys	GCA Ala	TTG Leu	ACT Thr	AAT Asn 135	GGA Gly	AGC Ser	ATT Ile	GAA Glu 140	GTC Val	CAA Gln	432
TGC Cys	ATG Met	ATA Ile 145	CCA Pro	GAG Glu	CAT His	TAC Tyr	AAC Asn	AGC Ser 150	CAT His	CGA Arg	GGA Gly	CTT Leu	ATA Ile 155	ATG Met	TAC Tyr	480
TCA Ser	TGG Trp	GAT Asp 160	TGT Cys	CCT Pro	ATG Met	GAG Glu	CAA Gln 165	TGT Cys	AAA Lys	CGT Arg	AAC Asn	TCA Ser 170	ACC Thr	AGT Ser	ATA Ile	528
TAT Tyr 175	TTT Phe	AAG Lys	ATG Met	GAA Glu	AAT Asn	GAT Asp 180	CTT Leu	CCA Pro	CAA Gln	AAA Lys	ATA Ile 185	CAG Gln	TGT Cys	ACT Thr	CTT Leu	576
AGC Ser 190	AAT Asn	CCA Pro	TTA Leu	TTT Phe	AAT Asn 195	ACA Thr	ACA Thr	TCA Ser	TCA Ser	ATC Ile 200	ATT Ile	TTG Leu	ACA Thr	ACC Thr	TGT Cys 205	624
ATC Ile	CCA Pro	AGC Ser	AGC Ser	GGT Gly 210	CAT His	TCA Ser	AGA Arg	CAC His	AGA Arg 215	TAT Tyr	GCA Ala	CTT Leu	ATA Ile	CCC Pro 220	ATA Ile	672
CCA Pro	TTA Leu	GCA Ala	GTA Val 225	ATT Ile	ACA Thr	ACA Thr	TGT Cys	ATT Ile 230	GTG Val	CTG Leu	TAT Tyr	ATG Met	AAT Asn 235	GGT Gly	ATT Ile	720
CTG Leu	AAA Lys 240	TGT Cys	GAC Asp	AGA Arg	AAA Lys	CCA Pro	GAC Asp 245	AGA Arg	ACC Thr	AAC Asn	TCC Ser	AAT Asn 250	TGATTGGTAA			769
CAGAAGATGA AGACAACAGC ATAACTAAAT TATTTTAAAA ACTAAAAAGC CATCTGATTT																829
CTCATTGAG TATTACAATT TTTGAACAAC TGTTGGAAAT GTAACCTGAA GCAGCTGCTT																889
TAAGAAGAAA TACCCACTAA CAAAGAACAA GCATTAGTTT TGGCTGTCAT CAACCTATTA																949
TATGACTAGG TGCTTGCTTT TTTTGTGTCAGT AAATTGTTTT TACTGATGAT GTAGATACTT																1009
TTGTAAATAA ATGTAAATAT GTACACAAGT G																1040

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 250 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Mct	Val	Ala	Gly	Scr	Asp	Ala	Gly	Arg	Ala	Leu	Gly	Val	Leu	Scr	Val
1				5					10					15	
Val	Cys	Leu	Leu	His	Cys	Phe	Gly	Phe	Ile	Scr	Cys	Phe	Scr	Gln	Gln
			20					25					30		
Ile	Tyr	Gly	Val	Val	Tyr	Gly	Asn	Val	Thr	Phe	His	Val	Pro	Scr	Asn
		35					40					45			
Val	Pro	Leu	Lys	Glu	Val	Leu	Trp	Lys	Lys	Gln	Lys	Asp	Lys	Val	Ala
	50					55					60				
Glu	Leu	Glu	Asn	Scr	Glu	Phe	Arg	Ala	Phe	Scr	Scr	Phe	Lys	Asn	Arg
65					70					75					80
Val	Tyr	Leu	Asp	Thr	Val	Scr	Gly	Scr	Leu	Thr	Ile	Tyr	Asn	Leu	Thr
				85					90					95	
Scr	Scr	Asp	Glu	Asp	Glu	Tyr	Glu	Mct	Glu	Scr	Pro	Asn	Ile	Thr	Asp
			100					105					110		
Thr	Mct	Lys	Phe	Phe	Leu	Tyr	Val	Leu	Glu	Scr	Leu	Pro	Scr	Pro	Thr
		115					120					125			
Leu	Thr	Cys	Ala	Leu	Thr	Asn	Gly	Scr	Ile	Glu	Val	Gln	Cys	Mct	Ile
	130					135					140				
Pro	Glu	His	Tyr	Asn	Scr	His	Arg	Gly	Leu	Ile	Mct	Tyr	Scr	Trp	Asp
145					150					155					160
Cys	Pro	Mct	Glu	Gln	Cys	Lys	Arg	Asn	Scr	Thr	Scr	Ile	Tyr	Phe	Lys
				165					170					175	
Mct	Glu	Asn	Asp	Leu	Pro	Gln	Lys	Ile	Gln	Cys	Thr	Leu	Scr	Asn	Pro
			180					185					190		
Leu	Phe	Asn	Thr	Thr	Scr	Scr	Ile	Ile	Leu	Thr	Thr	Cys	Ile	Pro	Scr
		195					200					205			
Scr	Gly	His	Scr	Arg	His	Arg	Tyr	Ala	Leu	Ile	Pro	Ile	Pro	Leu	Ala
	210					215					220				
Val	Ile	Thr	Thr	Cys	Ile	Val	Leu	Tyr	Mct	Asn	Gly	Ile	Leu	Lys	Cys
225					230					235					240
Asp	Arg	Lys	Pro	Asp	Arg	Thr	Asn	Scr	Asn						
				245					250						

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 863 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(i x) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 18..737

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCGGCCGCGC	ACGAGCC	ATG	GTT	GCT	GGG	AGC	GAC	GCG	GGG	CGG	GCC	CTG				50
		Mct	Val	Ala	Gly	Scr	Asp	Ala	Gly	Arg	Ala	Leu				
		1				5						10				
GGG	GTC	CTC	AGC	GTG	GTC	TGC	CTG	CTG	CAC	TGC	TTT	GGT	TTC	ATC	AGC	98
Gly	Val	Leu	Scr	Val	Val	Cys	Leu	Leu	His	Cys	Phe	Gly	Phe	Ile	Scr	
			15				20					25				
TGT	TTT	TCC	CAA	CAA	ATA	TAT	GGT	GTT	GTG	TAT	GGG	AAT	GTA	ACT	TTC	146
Cys	Phe	Scr	Gln	Gln	Ile	Tyr	Gly	Val	Val	Tyr	Gly	Asn	Val	Thr	Phe	

-continued

30			35			40										
CAT His	GTA Val 45	CCA Pro	AGC Ser	AAT Asn	GTG Val	CCT Pro	TTA Leu	AAA Lys	GAG Glu	GTC Val	CTA Leu 55	TGG Trp	AAA Lys	AAA Lys	CAA Gln	194
AAG Lys	GAT Asp 60	AAA Lys	GTT Val	GCA Ala	GAA Glu 65	CTG Leu	GAA Glu	AAT Asn	TCT Ser	GAA Glu 70	TTC Phe	AGA Arg	GCT Ala	TTC Phe	TCA Ser 75	242
TCT Ser	TTT Phe	AAA Lys	AAT Asn	AGG Arg 80	GTT Val	TAT Tyr	TTA Leu	GAC Asp	ACT Thr 85	GTG Val	TCA Ser	GGT Gly	AGC Ser	CTC Leu 90	ACT Thr	290
ATC Ile	TAC Tyr	AAC Asn	TTA Leu 95	ACA Thr	TCA Ser	TCA Ser	GAT Asp	GAA Glu 100	GAT Asp	GAG Glu	TAT Tyr	GAA Glu	ATG Met 105	GAA Glu	TCG Ser	338
CCA Pro	AAT Asn	ATT Ile 110	ACT Thr	GAT Asp	ACC Thr	ATG Met	AAG Lys 115	TTC Phe	TTT Phe	CTT Leu	TAT Tyr	GTG Val 120	CTT Leu	GAG Glu	TCT Ser	386
CTT Leu	CCA Pro 125	TCT Ser	CCC Pro	ACA Thr	CTA Leu	ACT Thr 130	TGT Cys	GCA Ala	TTG Leu	ACT Thr	AAT Asn 135	GGA Gly	AGC Ser	ATT Ile	GAA Glu	434
GTC Val 140	CAA Gln	TGC Cys	ATG Met	ATA Ile	CCA Pro 145	GAG Glu	CAT His	TAC Tyr	AAC Asn	AGC Ser 150	CAT His	CGA Arg	GGA Gly	CTT Leu	ATA Ile 155	482
ATG Met	TAC Tyr	TCA Ser	TGG Trp	GAT Asp 160	TGT Cys	CCT Pro	ATG Met	GAG Glu	CAA Gln 165	TGT Cys	AAA Lys	CGT Arg	AAC Asn	TCA Ser 170	ACC Thr	530
AGT Ser	ATA Ile	TAT Tyr	TTT Phe 175	AAG Lys	ATG Met	GAA Glu	AAT Asn	GAT Asp 180	CTT Leu	CCA Pro	CAA Gln	AAA Lys	ATA Ile 185	CAG Gln	TGT Cys	578
ACT Thr	CTT Leu	AGC Ser 190	AAT Asn	CCA Pro	TTA Leu	TTT Phe	AAT Asn 195	ACA Thr	ACA Thr	TCA Ser	TCA Ser	ATC Ile 200	ATT Ile	TTG Leu	ACA Thr	626
ACC Thr	TGT Cys 205	ATC Ile	CCA Pro	AGC Ser	AGC Ser	GGT Gly 210	CAT His	TCA Ser	AGA Arg	CAC His	AGA Arg 215	TAT Tyr	GCA Ala	CTT Leu	ATA Ile	674
CCC Pro 220	ATA Ile	CCA Pro	TTA Leu	GCA Ala	GTA Val 225	ATT Ile	ACA Thr	ACA Thr	TGT Cys	ATT Ile 230	GTG Val	CTG Leu	TAT Tyr	ATG Met	AAT Asn 235	722
GGT Gly	ATG Met	TAT Tyr	GCT Ala	TTT Phe 240	TAAAACAAAA TAGTTTGAAA ACTTGCATTG TTTTCCAAAG										777	
GTCAGAAAAT AGTTTAAGGA TGAAAATAAA GTTTGAAATT TTAGACATTT GAAAAAAAAA																837
AAAAAAAAAA AAAAAAAAAAGC GGCCGC																863

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 240 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met 1	Val	Ala	Gly	Ser	Asp	Ala	Gly	Arg	Ala	Leu	Gly	Val	Leu	Ser	Val	15
Val	Cys	Leu	Leu	His	Cys	Phe	Gly	Phe	Ile	Ser	Cys	Phe	Ser	Gln	Gln	20
Ile	Tyr	Gly	Val	Val	Tyr	Gly	Asn	Val	Thr	Phe	His	Val	Pro	Ser	Asn	35
Val	Pro	Leu	Lys	Glu	Val	Leu	Trp	Lys	Lys	Gln	Lys	Asp	Lys	Val	Ala	40

-continued

50					55					60					
Glu	Leu	Glu	Asn	Ser	Glu	Phe	Arg	Ala	Phe	Ser	Ser	Phe	Lys	Asn	Arg
65					70					75					80
Val	Tyr	Leu	Asp	Thr	Val	Ser	Gly	Ser	Leu	Thr	Ile	Tyr	Asn	Leu	Thr
				85					90					95	
Ser	Ser	Asp	Glu	Asp	Glu	Tyr	Glu	Met	Glu	Ser	Pro	Asn	Ile	Thr	Asp
			100					105					110		
Thr	Met	Lys	Phe	Phe	Leu	Tyr	Val	Leu	Glu	Ser	Leu	Pro	Ser	Pro	Thr
		115					120					125			
Leu	Thr	Cys	Ala	Leu	Thr	Asn	Gly	Ser	Ile	Glu	Val	Gln	Cys	Met	Ile
	130					135					140				
Pro	Glu	His	Tyr	Asn	Ser	His	Arg	Gly	Leu	Ile	Met	Tyr	Ser	Trp	Asp
145					150					155					160
Cys	Pro	Met	Glu	Gln	Cys	Lys	Arg	Asn	Ser	Thr	Ser	Ile	Tyr	Phe	Lys
				165					170					175	
Met	Glu	Asn	Asp	Leu	Pro	Gln	Lys	Ile	Gln	Cys	Thr	Leu	Ser	Asn	Pro
			180					185					190		
Leu	Phe	Asn	Thr	Thr	Ser	Ser	Ile	Ile	Leu	Thr	Thr	Cys	Ile	Pro	Ser
		195					200					205			
Ser	Gly	His	Ser	Arg	His	Arg	Tyr	Ala	Leu	Ile	Pro	Ile	Pro	Leu	Ala
	210					215					220				
Val	Ile	Thr	Thr	Cys	Ile	Val	Leu	Tyr	Met	Asn	Gly	Met	Tyr	Ala	Phe
225					230					235					240

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCTTTTAAA GGCACATACA CAACACCATA

30

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AACTTTATCC TTTTGATTGC TTGGTACATG

30

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCTCTGAAT TCAGATTTTT TCCATAGGAC

3 0

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAAATAAACC CTATTATTTT CCAGTTCTGC

3 0

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

G TAGATAGTG AGGCTTTTAA AAGATGAGAA

3 0

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

A TACTCATCT TCATCACCTG ACACAGTGTC

3 0

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGTATCAGTA ATATTTGATG ATGTTAAGTT

3 0

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGACTCAAGC ACATATGGCG ATTCCATTTC

3 0

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAATGCACAA GTTAGAAGAA AGAACTTCAT

3 0

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CATGCATTGG ACTTCTGTGG GAGATGGAAG

3 0

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCCTCGATGG CTGTTAATGC TTCCATTAGT

3 0

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CATAGGACAA TCCCAGTAAT GCTCTGGTAT

3 0

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TATACTGGTT GAGTTTGAGT ACATTATAAG

3 0

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTGTGGAAGA TCATTACGTT TACATTGCTC

3 0

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TAATGGATTG CTAAGTTCCA TCTTAAAATA

3 0

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TGTCAAAATG ATTGAAGTAC ACTGTATTTT

3 0

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

T C T T G A A T G A C C G C T T G A T G T T G T A T T A A A

3 0

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

A T A C T C A T C T T C A T C A T A C A C A A C A C C A T A

3 0

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

C A T A G G A C A A T C C C A T G A T G A T G T T A A G T T

3 0

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

T C T T G A A T G A C C G C T T G A G T A C A T T A T A A G

3 0

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

S e r L e u T h r I l e T y r A s n L e u T h r S e r S e r A s p G l u A s p G l u T y r G l u
 1 5 1 0 1 5

M e t G l u S e r P r o A s n I l e T h r A s p T h r M e t L y s P h e P h e L e u T y r V a l
 2 0 2 5 3 0

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(i x) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 6..23

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TCGTC GAC AAA ACT CAC ACA TGC C

24

Asp Lys Thr His Thr Cys
 1 5

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Asp Lys Thr His Thr Cys
 1 5

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GTAAATGAGT GCGGCGGCCG CCAA

24

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 115 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCGGCCGCGG TCCAACCACC AATCTCAAAG CTTGGTACCC GGAATTCAG ATCTGCAGCA

60

TGCTCGAGCT CTAGATATCG ATTCCATGGA TCCTCACATC CCAATCCGCG GCCGC

115

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(i x) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 13..33

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GAGGCGGCCG CC ATG GTT GCT GGG AGC GAC GCG
 Met Val Ala Gly Ser Asp Ala
 1 5

3 3

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Val Ala Gly Ser Asp Ala
 1 5

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(j i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AAGTCGACAT AAAGAAAGAA CTTCAT

2 6

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TCGACGCGGC CGCG

1 4

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1050 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(i x) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1041

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ATG Met 1	GTT Val	GCT Ala	GGG Gly	AGC Ser 5	GAC Asp	GCG Ala	GGG Gly	CGG Arg	GCC Ala 10	CTG Leu	GGG Gly	GTC Val	CTC Leu	AGC Ser 15	GTG Val	48
GTC Val	TGC Cys	CTG Leu	CTG Leu 20	CAC His	TGC Cys	TTT Phe	GGT Gly	TTC Phe 25	ATC Ile	AGC Ser	TGT Cys	TTT Phe	TCC Ser 30	CAA Gln	CAA Gln	96
ATA Ile	TAT Tyr	GGT Gly 35	GTT Val	GTG Val	TAT Tyr	GGG Gly	AAT Asn 40	GTA Val	ACT Thr	TTC Phe	CAT His	GTA Val 45	CCA Pro	AGC Ser	AAT Asn	144
GTG Val	CCT Pro 50	TTA Leu	AAA Lys	GAG Glu	GTC Val	CTA Leu 55	TGG Trp	AAA Lys	AAA Lys	CAA Gln	AAG Lys 60	GAT Asp	AAA Lys	GTT Val	GCA Ala	192
GAA Glu 65	CTG Leu	GAA Glu	AAT Asn	TCT Ser	GAA Glu 70	TTC Phe	AGA Arg	GCT Ala	TTC Phe	TCA Ser 75	TCT Ser	TTT Phe	AAA Lys	AAT Asn	AGG Arg 80	240
GTT Val	TAT Tyr	TTA Leu	GAC Asp	ACT Thr 85	GTG Val	TCA Ser	GGT Gly	AGC Ser	CTC Leu 90	ACT Thr	ATC Ile	TAC Tyr	AAC Asn 95	TTA Leu	ACA Thr	288
TCA Ser	TCA Ser	GAT Asp	GAA Glu 100	GAT Asp	GAG Glu	TAT Tyr	GAA Glu 105	ATG Met	GAA Glu	TCG Ser	CCA Pro	AAT Asn 110	ATT Ile	ACT Thr	GAT Asp	336
ACC Thr	ATG Met	AAG Lys 115	TTC Phe	TTT Phe	CTT Leu	TAT Tyr	GTC Val 120	GAC Asp	AAA Lys	ACT Thr	CAC His	ACA Thr 125	TGC Cys	CCA Pro	CCG Pro	384
TGC Cys 130	CCA Pro	GCA Ala	CCT Pro	GAA Glu	CTC Leu	CTG Leu 135	GGG Gly	GGA Gly	CCG Pro	TCA Ser	GTC Val 140	TTC Phe	CTC Leu	TTC Phe	CCC Pro	432
CCA Pro 145	AAA Lys	CCC Pro	AAG Lys	GAC Asp	ACC Thr 150	CTC Leu	ATG Met	ATC Ile	TCC Ser	CGG Arg 155	ACC Thr	CCT Pro	GAG Glu	GTC Val 160	ACA Thr 160	480
TGC Cys	GTG Val	GTG Val	GTG Val	GAC Asp 165	GTG Val	AGC Ser	CAC His	GAA Glu	GAC Asp 170	CCT Pro	GAG Glu	GTC Val	AAG Lys	TTC Phe 175	AAC Asn 175	528
TGG Trp	TAC Tyr	GTG Val	GAC Asp 180	GGC Gly	GTG Val	GAG Glu	GTG Val	CAT His 185	AAT Asn	GCC Ala	AAG Lys	ACA Thr	AAG Lys 190	CCG Pro	CGG Arg	576
GAG Glu	GAG Glu	CAG Gln 195	TAC Tyr	AAC Asn	AGC Ser	ACG Thr	TAC Tyr 200	CGG Arg	GTG Val	GTC Val	AGC Ser	GTC Val 205	CTC Leu	ACC Thr	GTC Val	624
CTG Leu	CAC His 210	CAG Gln	GAC Asp	TGG Trp	CTG Leu	AAT Asn 215	GGC Gly	AAG Lys	GAG Glu	TAC Tyr	AAG Lys 220	TGC Cys	AAG Lys	GTC Val	TCC Ser	672
AAC Asn 225	AAA Lys	GCC Ala	CTC Leu	CCA Pro	GCC Ala 230	CCC Pro	ATC Ile	GAG Glu	AAA Lys	ACC Thr 235	ATC Ile	TCC Ser	AAA Lys	GCC Ala 240	AAA Lys 240	720
GGG Gly	CAG Gln	CCC Pro	CGA Arg	GAA Glu 245	CCA Pro	CAG Gln	GTG Val	TAC Tyr	ACC Thr 250	CTG Leu	CCC Pro	CCA Pro	TCC Ser	CGG Arg 255	GAT Asp	768
GAG Glu	CTG Leu	ACC Thr	AAG Lys 260	AAC Asn	CAG Gln	GTC Val	AGC Ser	CTG Leu 265	ACC Thr	TGC Cys	CTG Leu	GTC Val	AAA Lys 270	GGC Gly	TTC Phe	816
TAT Tyr	CCC Pro	AGC Ser	GAC Asp	ATC Ile	GCC Ala	GTG Val	GAG Glu 280	TGG Trp	GAG Glu	AGC Ser	AAT Asn 285	GGG Gly	CAG Gln	CCG Pro	GAG Glu	864
AAC Asn 290	AAC Asn	TAC Tyr	AAG Lys	ACC Thr	ACG Thr	CCT Pro 295	CCC Pro	GTG Val	CTG Leu	GAC Asp	TCC Ser 300	GAC Asp	GGC Gly	TCC Ser	TTC Phe	912
TTC Phe	CTC Leu	TAC Tyr	AGC Ser	AAG Lys	CTC Leu	ACC Thr	GTG Val	GAC Asp	AAG Lys	AGC Ser	AGG Arg	TGG Trp	CAG Gln	CAG Gln	GGG Gly	960

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305	310					315					320					
AAC	GTC	TTC	TCA	TGC	TCC	GTG	ATG	CAT	GAG	GCT	CTG	CAC	AAC	CAC	TAC	1008
Asn	Val	Phe	Scr	Cys	Scr	Val	Mct	His	Glu	Ala	Leu	His	Asn	His	Tyr	
				325					330					335		
ACG	CAG	AAG	AGC	CTC	TCC	CTG	TCT	CCG	GGT	AAA	TGAGTGCGG					1050
Thr	Gln	Lys	Scr	Leu	Scr	Leu	Scr	Pro	Gly	Lys						
			340					345								

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 347 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Mct	Val	Ala	Gly	Scr	Asp	Ala	Gly	Arg	Ala	Leu	Gly	Val	Leu	Scr	Val
1				5					10					15	
Val	Cys	Leu	Leu	His	Cys	Phe	Gly	Phe	Ile	Scr	Cys	Phe	Scr	Gln	Gln
			20					25						30	
Ile	Tyr	Gly	Val	Val	Tyr	Gly	Asn	Val	Thr	Phe	His	Val	Pro	Scr	Asn
		35					40					45			
Val	Pro	Leu	Lys	Glu	Val	Leu	Trp	Lys	Lys	Gln	Lys	Asp	Lys	Val	Ala
	50					55					60				
Glu	Leu	Glu	Asn	Scr	Glu	Phe	Arg	Ala	Phe	Scr	Scr	Phe	Lys	Asn	Arg
65				70						75					80
Val	Tyr	Leu	Asp	Thr	Val	Scr	Gly	Scr	Leu	Thr	Ile	Tyr	Asn	Leu	Thr
				85					90					95	
Scr	Scr	Asp	Glu	Asp	Glu	Tyr	Glu	Mct	Glu	Scr	Pro	Asn	Ile	Thr	Asp
			100					105					110		
Thr	Mct	Lys	Phe	Phe	Leu	Tyr	Val	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro
		115					120					125			
Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Scr	Val	Phe	Leu	Phe	Pro
	130					135					140				
Pro	Lys	Pro	Lys	Asp	Thr	Leu	Mct	Ile	Scr	Arg	Thr	Pro	Glu	Val	Thr
145				150						155					160
Cys	Val	Val	Val	Asp	Val	Scr	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn
				165					170					175	
Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg
			180					185						190	
Glu	Glu	Gln	Tyr	Asn	Scr	Thr	Tyr	Arg	Val	Val	Scr	Val	Leu	Thr	Val
		195					200					205			
Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Scr
	210					215					220				
Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Scr	Lys	Ala	Lys
225				230						235					240
Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Scr	Arg	Asp
				245					250					255	
Glu	Leu	Thr	Lys	Asn	Gln	Val	Scr	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe
			260					265					270		
Tyr	Pro	Scr	Asp	Ile	Ala	Val	Glu	Trp	Glu	Scr	Asn	Gly	Gln	Pro	Glu
		275					280					285			
Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	Asp	Scr	Asp	Gly	Scr	Phe
290						295					300				
Phe	Leu	Tyr	Scr	Lys	Leu	Thr	Val	Asp	Lys	Scr	Arg	Trp	Gln	Gln	Gly

3 0 5		3 1 0		3 1 5		3 2 0									
A s n	V a l	P h e	S e r	C y s	S e r	V a l	M e t	H i s	G l u	A l a	L e u	H i s	A s n	H i s	T y r
				3 2 5					3 3 0					3 3 5	
T h r	G l n	L y s	S e r	L e u	S e r	L e u	S e r	P r o	G l y	L y s					
			3 4 0					3 4 5							

We claim:

1. A polypeptide having the amino acid sequence: X₁-X₂- (SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ if present, is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val; said polypeptide being capable of binding to CD2.

2. The polypeptide according to claim 1 wherein,

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 10 amino acids of the sequence (SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser;

said polypeptide being capable of binding to CD2.

3. A polypeptide selected from the group consisting of polypeptides having the amino acid sequences: amino acids 29-120 of SEQ ID NO:10, amino acids 29-108 of SEQ ID NO:10, amino acids 48-108 of SEQ ID NO:10, and SEQ ID NO:7.

4. A polypeptide having the amino acid sequence (SEQ ID NO:7): Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr.

5. A multimeric protein capable of binding to CD2 comprising two or more polypeptides according to any one of claims 1-4.

6. A method for labeling CD2⁺ cells or proteins containing the LFA-3 binding domain of CD2, said method comprising the step of incubating the CD2⁺ cells or proteins containing the LFA-3 binding domain of CD2 with a polypeptide according to any one of claims 1-4.

7. A pharmaceutical composition comprising a polypeptide according to any one of claims 1-4 and a pharmaceutically acceptable carrier.

8. A diagnostic kit for detecting the presence of CD2⁺ cells or LFA-3-binding proteins comprising a polypeptide

10 according to any one of claims 1-4 together with one or more reagents necessary for detecting the binding of CD2⁺ cells or LFA-3-binding proteins to the polypeptide.

9. An isolated DNA encoding a polypeptide selected from the group consisting of:

15 (a) a polypeptide having the amino acid sequence: X₁-X₂- (SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ if present, is hydrogen or methionyl;

20 X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

25 Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;

said polypeptide being capable of binding to CD2;

(b) a polypeptide having the amino acid sequence: X₁-X₂- (SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

40 X₁ if present, is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

45 Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 10 amino acids of the sequence (SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser;

said polypeptide being capable of binding to CD2;

(c) a polypeptide having the amino acid sequence of amino acids 29-120 of SEQ ID No:10;

(d) a polypeptide having the amino acid sequence of amino acids 29-108 of SEQ ID No:10;

(e) a polypeptide having the amino acid sequence of amino acids 48-108 of SEQ ID No:10; and

(f) a polypeptide having the amino acid sequence of (SEQ ID No:7): Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr.

65 10. A recombinant DNA molecule comprising a DNA according to claim 9 and an expression control sequence,

wherein the expression control sequence is operatively linked to the DNA.

11. A method for producing a CD2 binding protein comprising the steps of culturing a host cell transformed with the recombinant DNA molecule of claim 10 and recovering the protein encoded thereby.

12. A host cell transformed with the recombinant DNA molecule according to claim 10.

13. An isolated DNA encoding the CD2-binding domain of LFA-3 consisting of:

(5') (SEQ ID NO:6) AATAGGGTTT ATTAGACAC TGTGTCAGGT (3').

14. A recombinant DNA molecule comprising a DNA according to claim 13 and an expression control sequence, wherein the expression control sequence is operatively linked to the DNA.

15. An isolated DNA consisting of a DNA encoding the CD2-binding domain of LFA-3 selected from the group consisting of the DNA inserts of pPYM57, pPMDRM54-6, pPMDRM55-9, pPMDRM56-C, pPMDRM58-15, pPYM63-4, pPYM65-8, pPMDRM100-4, pPMDRM101-1, pPMDRM102-8, and pPMDRM3-10.

16. A recombinant DNA molecule comprising a DNA according to claim 15 and an expression control sequence, wherein the expression control sequence is operatively linked to the DNA.

17. A method for producing a CD2 binding protein comprising the steps of culturing a host cell transformed with the recombinant DNA molecule of claim 14 or 16 and recovering the protein encoded thereby.

18. A host cell transformed with the recombinant DNA molecule according to claim 14 or 16.

19. A fusion protein, having an amino terminal region consisting of the amino acid sequence of a polypeptide selected from the group consisting of;

(a) a polypeptide having the amino acid sequence: X₁-X₂- (SEQ. ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ if present, is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;

said polypeptide being capable of binding to CD2;

(b) a polypeptide having the amino acid sequence: X₁-X₂- (SEQ. ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ if present, is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp

Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 10 amino acids of the sequence (SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser;

said polypeptide being capable of binding to CD2;

(c) a polypeptide having the amino acid sequence of amino acids 29-120 of SEQ ID No:10;

(d) a polypeptide having the amino acid sequence of amino acids 29-108 of SEQ ID No:10;

(e) a polypeptide having the amino acid sequence of amino acids 48-108 of SEQ ID No:10; and

(f) a polypeptide having the amino acid sequence of (SEQ ID No:7): Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr;

and having a carboxy terminal region comprising a domain of a protein or polypeptide other than LFA-3.

20. The fusion protein according to claim 19 wherein the carboxy terminal region comprises at least a portion of the Fc region of an immunoglobulin.

21. The fusion protein according to claim 20 wherein the portion of the Fc region of an immunoglobulin comprises a hinge region and C_H2 and C_H3 constant domains.

22. The fusion protein according to claim 20 wherein the portion of the Fc region comprises a portion of a hinge region that is capable of forming intermolecular disulfide bonds and C_H2 and C_H3 constant domains.

23. The fusion protein according to claim 22 which is amino acids 29-347 of SEQ ID NO:43.

24. An isolated DNA coding for a fusion protein according to any one of claims 19-23.

25. A recombinant DNA molecule comprising the DNA according to claim 24 and an expression control sequence, wherein the expression control sequence is operatively linked to the DNA.

26. A method for producing a CD2 binding protein comprising the steps of culturing a host cell transformed with the recombinant DNA molecule of claim 25 and recovering the protein encoded thereby.

27. A host cell transformed with the recombinant DNA molecule according to claim 25.

28. The recombinant DNA molecule, according to claim 25 selected from the group consisting of plasmid pSAB152 and plasmid pMDR(92)Ig-3.

29. A method for producing a CD2 binding protein comprising the steps of culturing a host cell transformed with the recombinant DNA molecule of claim 28 and recovering the protein encoded thereby.

30. A host cell transformed with the recombinant DNA molecule according to claim 28.

31. A multimeric protein capable of binding to CD2 comprising two or more fusion proteins according to any one of claims 19-23.

32. A method for labeling CD2⁺ cells or proteins containing the LFA-3 binding domain of CD2, said method comprising the step of incubating the CD2⁺ cells or proteins containing the LFA-3 binding domain of CD2 with a fusion protein according to any one of claims 19-23.

33. A pharmaceutical composition comprising a fusion protein according to any one of claims 19-23, and a pharmaceutically acceptable carrier.

34. A diagnostic kit comprising a fusion protein according to any one of claims 19-23 together with one or more reagents necessary for detecting the binding of CD2⁺ cells or LFA-3-binding proteins to the fusion protein.

35. A multimeric protein capable of binding to CD2 comprising: (a) one or more polypeptides having the amino acid sequence: X₁-X₂-(SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ if present, is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;

said polypeptides being capable of binding to CD2; and

(b) one or more fusion proteins having an amino terminal region consisting of the amino acid sequence of a polypeptide having the amino acid sequence: X₁-X₂-

(SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X₁ if present, is hydrogen or methionyl;

X₂, if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;

said polypeptide being capable of binding to CD2; and having a carboxy terminal region comprising a domain of a protein or polypeptide other than LFA-3.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

(12) CERTIFICATE EXTENDING PATENT TERM
UNDER 35 U.S.C. § 156

(68) PATENT NO. : 5,547,853
(45) ISSUED : August 20, 1996
(75) INVENTOR : Barbara P. Wallner, et al.
(73) PATENT OWNER : Biogen Idec MA, Inc.
(95) PRODUCT : AMEVIVE® (alefacept)

This is to certify that an application under 35 U.S.C. § 156 has been filed in the United States Patent and Trademark Office, requesting extension of the term of U.S. Patent No. 5,547,853 based upon the regulatory review of the product AMEVIVE® (alefacept) by the Food and Drug Administration. Since it appears that the requirements of the law have been met, this certificate extends the term of the patent for the period of

(94) 1,259 days

from August 20, 2013, the original expiration date of the patent, subject to the payment of maintenance fees as provided by law, with all rights pertaining thereto as provided by 35 U.S.C. § 156(b).



I have caused the seal of the United States Patent and Trademark Office to be affixed this 1st day of May 2006.


Jon W. Dudas
Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office